THE EFFECT OF MECHANICAL TENSION UPON THE POLARITY OF GROWING FIBROBLASTS.

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Plate 4.

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In the healing of a sterile surgical skin incision the newly formed fibroblasts show a definite polarity which leads them to grow across the wound parallel to the surface and to all practical purposes parallel to each other. The cause of this polarity is not obvious in sections of such growing tissue. However, other pathological new growths of connective tissue give a hint that mechanical tension plays a part in the process. Thus in an organizing pericardial exudate, fibroblasts grow out parallel to the newly formed capillaries and perpendicular to the surface, and eventually come to lie parallel to the surface unless an adhesion is formed between the two pericardial surfaces, when they maintain their original position parallel to the vessels. The experiments here reported are an attempt to establish the effect of mechanical tension upon the polarity of growing fibroblasts.

For the purposes of the experiments incisions were made into the thick corium of the back of anesthetized rabbits, with aseptic precautions. In order to secure variation in the direction of tension upon the healing wound, a wire fly screen saddle was fitted close to the back, permitting deep sutures, placed at selected points, to be brought through the screen at any desired point and tied above so as to maintain pull in the direction wished. At varying intervals such wounds were excised, fixed in Zenker's fluid, and cut in serial sections parallel to the surface. This procedure was adopted after it was found that strips of adhesive tape would not adhere to rabbit's skin with sufficient firmness to maintain the desired tension.

In Rabbit 4, two incisions about 2 cm. in length were made, one at either side of the midline of the back. In the first, the natural tendency of the skin edges to gape was exaggerated by sutures placed at either side of the wound with the tension applied at right angles to the line of the incision. In the second, the sutures were placed beyond the ends of the incision with tension applied in the line of the cut. 4 days later, the wounds were excised and sectioned. Examination of the

sections showed that in each the main line of fibroblastic growth was in the direction of the applied tension. Repetitions of the experiment gave the same result, and Figs. 1 and 2 show the difference in direction of growth in Rabbit 10, in which excision of the tissue was made 6 days after the primary incision.

In order to test further the effect of tension, a knife point incision of less than 5 mm. in length was made in the skin of the back and four pair of tension sutures arranged about the cut so as to give a simultaneous centrifugal pull perpendicular to the line of incision, parallel to the line, and along the 45° diagonals. The wound which had been stretched into a small oval ulcer was excised at the end of 7 days and sectioned parallel to the surface. Fig. 3 is a photograph taken from as near the centre as possible of this wound and it shows a well marked radial tendency of growth on the part of the fibroblasts.

These experiments seem to indicate that in connective tissue growing under mechanical tension the spindle-shaped fibroblasts will arrange themselves with their long axis parallel to the line of pull. That this is not simply an elongation of the protoplasm of the cell in a certain direction, but is the expression of a definite polarity in the cell is indicated by a study of the mitotic figures occurring in the sections. In the dividing cells found in the tissues, the figures lie in such manner that a line connecting the poles of the achromatic spindle is in the line of tension, and thus the equatorial plane and the plane of cell division are perpendicular. The two daughter cells thus separate in the line of tension.

In view of the behavior of fibroblasts in granulation tissue, it seemed important to determine whether or not a shift in tension would cause fibroblasts to shift their polarity or position after it was once established. As is well known, in granulation tissue the new fibroblasts at first grow out parallel to the capillary sprouts, and thus, in the surface or youngest part of the granulation, lie perpendicular to the free surface. Deeper down in the granulation they lie in an oblique position and at the base in the older part of the tissue they lie perpendicular to the vessels and parallel to the surface, a position found in the ultimate scar tissue. To test the effect of change in tension, traction was applied perpendicular to the line of a skin incision for 2 days; these sutures were then cut and traction made by a second pair of sutures in the line of the incision and at right angles to the first tension. After 4 days, the tissue was excised and sectioned. Fibroblasts in the scar line were all arranged parallel to the line of incision

and to the line of last tension. While no cross fibroblasts were found in the scar line, it seemed possible that this result might be interpreted as indicating merely that the majority of the fibroblasts had been laid down after the shift in tension was made, and had obscured the few laid down earlier. In consequence, in a repetition of the experiment, the interval between the shift in tension and the excision of tissue was cut down from 4 days to 2. In sections from this tissue, while the general direction of the fibroblasts in the scar was parallel to the line of incision and of the last tension, yet many lay in a direction oblique to this line, and the bodies of others were definitely curved as if the cell were in the act of turning. This seemed to clear up any question that might have persisted concerning the result of the earlier experiment.

CONCLUSION.

These experiments seem to justify the conclusion that mechanical tension may determine the polarity of cell division and line of growth of developing fibroblasts, and further that a shift in tension may cause a shift in position of fibroblasts already formed.

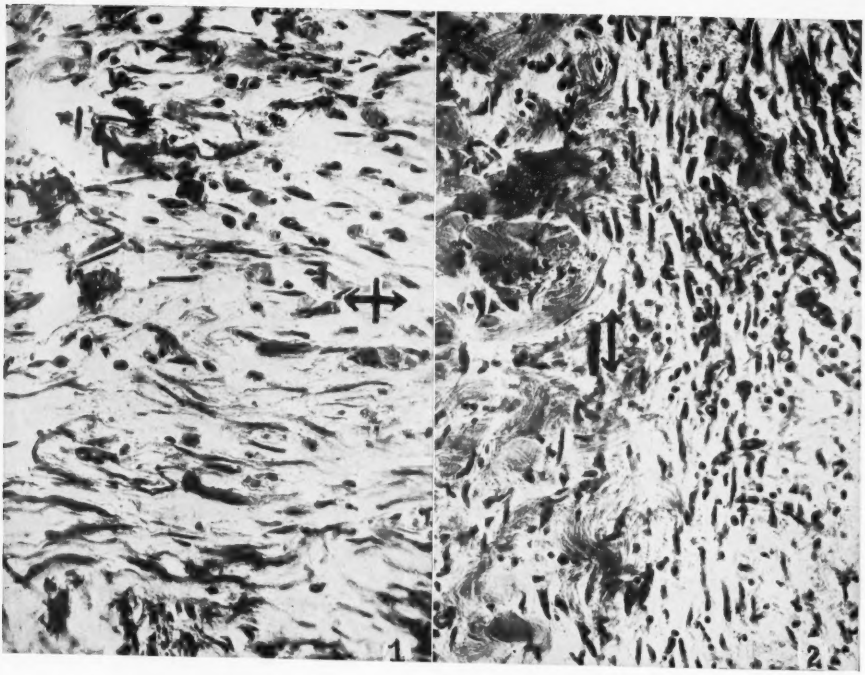
EXPLANATION OF PLATE 4.

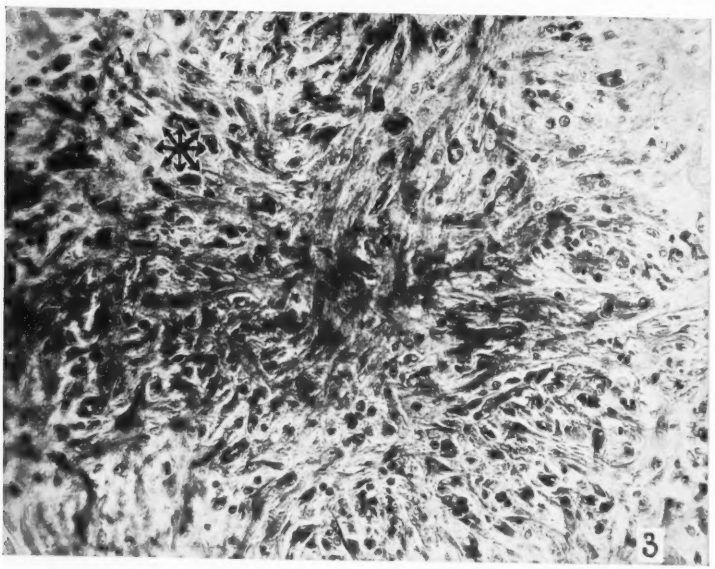
The figures are photomicrographs of healing wounds, and were made from sections cut in a plane parallel to the skin surface.

FIG. 1. Section of healing wound in which tension was made perpendicular to line of incision which is vertical in the illustration.

FIG. 2. Section of healing wound in which tension is parallel to the line of incision, which is likewise vertical in the illustration.

FIG. 3. Section of knife point incision with radial centrifugal tension.





(Bunting and Eades: Polarity of growing fibroblasts.)

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THE PHYSIOLOGICAL VARIATIONS IN RESISTANCE TO BILE FLOW TO THE INTESTINE.

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PLATE 5.

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The flow of bile, from its source within the liver to its issue into the duodenum, is greatly influenced by two structures situated along the path of flow. These, the musculature about the lower portion of the common duct and the gall bladder, not only effect the actual movement of bile but by their combined activities modify the character of the secretion as well. The common bile duct, after entering the wall of the duodenum, passes for a considerable distance through its muscular coat to enter the intestinal lumen at the ampulla of Vater. At this terminal portion is situated the sphincter of Oddi. This structure, aided perhaps by the musculature of the duodenum surrounding the lower portion of the common duct, exerts a pressure sufficient to maintain closure of the orifice of the duct during much of the time. Under these conditions the direction of the flow of bile is not continuously from the liver to the intestine but more often in the reverse direction, from the liver back into the gall bladder. Here, as has been shown in this laboratory,[1] the bile is concentrated with remarkable rapidity. The gall bladder is thus enabled to admit and store the bile coming to it during the usual digestive interim. Nearly 30 years ago Bruno,[2,3] working with "Pavlov biliary fistula" dogs,[4] noted that bile emerged from the intact ampulla only at long intervals of $1\frac{1}{2}$ to $2\frac{1}{4}$ hours, and in small amounts during fasting periods. According to

[1] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 47.

[2] Bruno, G. G., *Arch. sc. biol.*, 1899, vii, 87.

[3] Bruno, G. G., Dissertation, St. Petersburg, 1898, cited by Babkin.[5]

[4] Pavlov, J. P., *Ergebn. Physiol.*, 1902, i, 1. Abt., 246.

Babkin[5] this observation was confirmed by Klodnizki[6] and also by Boldyreff.[7]

The demonstration of the fact that bile need not enter the intestine save at long intervals of time has made it a matter of greater interest to study the conditions under which the escape of bile to the gut actually does take place. Though much work has already been done upon the theme[8] there is little information to be had that is not based on indirect methods of observation; and there is but slight accord among workers upon the problem.

In the present paper the alterations occurring under pathological circumstances in the resistance to the flow of bile into the intestine will be described. The observations were made upon healthy, unanesthetized animals. In a paper to follow, the influence of the gall bladder will be considered.

PREVIOUS LITERATURE.

Anatomically the sphincter of the common duct is a well defined structure. As far back as 1680, Glisson[9] suggested its existence but nearly two centuries elapsed before other investigators took up its study. In 1878 Gage[10] first described the presence of circular fibers surrounding the duct in the ampulla of Vater, but the sphincter is best known through the detailed work of Oddi.[11] In many animals and in man there is about the lower portion of the common duct a discrete ring of muscle, distinct from the surrounding duodenal musculature.[12–14]

The physiology of the musculature of the lower portion of the common duct has only recently received much attention.[8,15] Oddi[16] on the basis of his experi-

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[5] Babkin, B. P., Die äussere Sekretion der Verdauungsdrüsen, Berlin, 1914, 338 *et seq.*

[6] Klodnizki, Dissertation, St. Petersburg, 1902, cited by Babkin,[5] p. 341.

[7] Boldyreff, W. H., Dissertation, St. Petersburg, 1904, cited by Babkin,[5] p. 341.

[8] Mann, F. C., *Physiol. Rev.*, 1924, iv, 251.

[9] Glisson, F., Anatomia hepatis, 1681, quoted by Oddi.[11]

[10] Gage, S. H., *Am. Quart. Micr. J.*, 1878–79, i, 123.

[11] Oddi, R., *Arch. ital. biol.*, 1887, viii, 317.

[12] Hendrickson, W. F., *Bull. Johns Hopkins Hosp.*, 1898, ix, 221.

[13] Helly, *Arch mikr. Anat.*, 1899, liv, 614.

[14] Mann, F. C., *J. Lab. and Clin. Med.*, 1919–20, v, 107.

[15] Westphal, K., *Klin. Woch.*, 1924, iii, 1105.

[16] Oddi, R., *Sperimentale*, 1894, xlviii, 180, as abstracted in *Schmidt's Jahrb.*, 1895, ccxlv, 120.

ments claimed for the sphincter nervous control independent of that of the neighboring intestinal muscle, further describing a center for it in the spinal cord opposite the first lumbar vertebra. He measured the sphincter tonus by observing the height of a column of water supported in a tube tied in the common duct. Doyon determined in anesthetized animals the amount of fluid passing through the ampulla from a specially constructed manometer connected with the common duct.[17] He claimed for the sphincter a reaction on reflex stimulation only, with contraction upon irritation of the gastric mucosa with acetic acid and relaxation following electrical stimulation of the central end of the cut vagus or upon section of the medulla. He quoted the observation of Vulpian, that after "*piqûre*" of the medulla in rabbits, the duodenum is found, at autopsy, filled with dark brown bile.[18] Reach[19] studied the resistance offered to the passage of bile through the ampulla in a way similar to that of Doyon but used the completely isolated common duct immersed in warm Ringer solution. Bruno,[2] Klodnizki,[6] Boldyreff,[7] and much later Rost,[20] employing unanesthetized dogs with a duodenal fistula, observed intermittent gushes of bile from the ampulla, under a variety of circumstances, and deemed them indicative of a relaxation of the sphincter. These findings will be discussed in detail further on, as too will the recent work of Cole,[21] who found in anesthetized dogs a relationship between the resistance to the flow of bile through the lower common duct and the reaction of the gastric contents.

Recently the importance of the action of the sphincter of Oddi as apart from that of the duodenal musculature surrounding the lower end of the common duct, has been called in question.[22,23]

In the present work we have employed as a criterion of the activity of the musculature about the lower portion of the common duct the pressure necessary to force bile past it into the intestine. No attempt has been made to differentiate the activity of the duodenal musculature in this region from that of the sphincter. The circumstances of the observations have been especially favorable, involving as they have natural conditions in healthy, robust animals, with no disturbance of the biliary tract other than that incident to an intubation of the common duct. The pressure determinations, made long after operation, required no anesthetic since they produced not the slightest

[17] Doyon, M., *Arch. physiol. norm. et path.*, 1894, vi, series 5, 19.
[18] Doyon, M., *Arch. physiol. norm. et path.*, 1893, v, series 5, 683.
[19] Reach, F., *Zentr. Physiol.*, 1912–13, xxvi, 1318.
[20] Rost, F., *Mitt. Grenzgeb. Med. u. Chir.*, 1913, xxvi, 711.
[21] Cole, W. H., *Am. J. Physiol.*, 1925, lxxii, 39.
[22] Carlson, A. J., *J. Am. Med. Assn.*, 1925, lxxxv, 1468.
[23] Burget, G. E., *Am. J. Physiol.*, 1925, lxxiv, 583.

discomfort. No such observations on the unanesthetized animal have been made in the past. The above mentioned observations by workers in Pavlov's laboratory[2,6,7] were indirect in nature and were frequently complicated by difficulties inherent in the procedure. Under the circumstances of the Pavlov biliary fistula[4] the portion of the duodenal mucosa transplanted to the body surface and containing the sphincter undergoes a gradual atrophy and retraction. The biliary tract and especially the portion about the ampulla becomes infected and the flow of bile is frequently obstructed.

Technique.

Normal, quiet, healthy dogs were employed in which the common bile duct was intubated, under ether, in two directions, toward the liver and toward the ampulla of Vater, the duct being cut between.[24] The gall bladder was excluded by section of the cystic duct between ligatures. A diagram of this scheme of intubation, "altercursive intubation," has already been published.[24] By means of it the bile is brought to the surface and can either be collected there or turned back into the common duct at will to flow as usual through the ampulla of Vater into the duodenum.

In Fig. 1 is shown a dog with the "altercursive intubation" installed, but with the protective wrappings removed from the joints of the tubing. The tube nearer the animal's head leads to the cannula that receives bile from the liver, the other connects with the cannula directed toward the ampulla of Vater. The excellent healing about the tubes should be noted. By means of a clamp placed on the tubing one can direct the flow of bile into the bag for collection, or prevent it from entering with result that it returns to the common duct at a point not over $\frac{1}{2}$ cm. from where it left it. As the figure shows, the rubber balloon is supported in a light wicker basket which is held in place against the animal's side by adhesive strips. This in turn is covered by a muslin and canvas jacket which laces up the back and, while permitting freedom of motion to the animal, prevents it from dislodging the apparatus. Sterility of the tube system is maintained by collecting the bile under aseptic precautions.

During the manipulations incident to the experiments sterility of the system can be maintained only by the most scrupulous care. Manometers and tubes were ster-

[24] McMaster, P. D., and Elman, R., *J. Exp. Med.*, 1925, xli, 513.

ilized before use, and all joints protected with gauze sponges soaked in 5 per cent carbolic acid. Routine cultures of the bile and examinations of stained sediment from centrifuged specimens were made to discover the presence of organisms. Despite all precautions infection frequently occurred, not through the bile but as result of the repeated opening of the tube system to connect it with the manometer. Usually saprophytes such as did not change the character of the bile in important ways were the invading organisms. Nevertheless when this occurred experiments with the animal were at once abandoned as was done also whenever any obstruction developed in the cannulas, even a temporary one. In the present paper we consider only results that were obtained during the maintenance of sterile and unobstructive conditions within the biliary tracts of animals.

For the purpose of determining the resistance to the flow of bile into the intestine the tube leading to the lower common duct was temporarily disconnected at intervals and attached under aseptic precautions to a manometer filled with the animal's own uninfected bile. While the tests were in progress the liver bile was allowed to flow either into the balloon or into a sterile flask. In a number of experiments (as in that of Text-fig. 8) the manometer was temporarily substituted for the collecting balloon, with result that the pressure existing within the tube-duct system was registered. The latter arrangement is shown in Fig. 2. Under these circumstances, too, the tube draining liver bile could be clamped temporarily, with result that only the resistance offered to the flow of bile into the intestine was measured.

After a few repetitions of the procedure, at most, the dogs learned to stand quietly beside the manometer, for hours even, and they evidently enjoyed the attention bestowed upon them. The level of the common duct above the table on which they stood was roughly estimated by external measurements and the zero point of the manometer set at this level. Many pressure readings were taken from each animal daily, over long periods of time, and in each individual the zero point of the manometer was placed at the same height above the table as in the first determinations. The actual level of the common duct was determined at autopsy. The error from the presumptive level was so slight as to be negligible.

The resistance offered to the passage of bile into the intestine was tested by noting the height to which it was necessary to raise the column of bile or salt solution in the manometer, attached to the tube leading to the lower common duct, in order to start a flow. The height of this column when flow ceased was also noted. In one series of experiments we joined the lower common duct with a special manometer, to be described below, which was arranged to show the rate of bile flow into the intestine at a relatively constant pressure.

At the outset of these studies observations were made at random. Soon however an influence of the ingestion of food on the resistance offered within the lower common duct became evident to us. Thereafter, in the experiments which will now be described, feedings were

given at stated times and the measurements made in relation to these times. The food consisted in every case of a liberal mixture of lean meat and bread soaked in milk, 450 gm. of the former to each kilo of the latter. The dogs were allowed to eat until satiated and the food was then removed. Special attention was paid to the influence of incidental body movements of the animals during the observations. In most cases the dog stood quietly for long periods with only occasional turning movements of the body, as it looked from one part of the room to the other. Since many duplicate observations were made at one time the influence of such body movements could be tested. It was found to be very slight.

The Normal Resistance to the Flow of Bile into the Intestine.

In the past numerous workers have measured how high a column of water had to be in a tube connected with the common duct in order to start a flow through the common duct. Widely divergent values ranging from 80 mm. to 600 mm. have been quoted.[8] The workers mentioned made their measurements on the anesthetized dog, however, and they had also to reckon with the influence of trauma incident to a laparotomy. No mention has been made in many of the studies of any time relation to the giving of food.

We have tested the resistance offered to the flow of bile through the lower common duct in eighteen dogs appropriately intubated. A value for the "normal" pressure was found that was uniform within narrow limits though subject to alterations from feeding and digestion. In scores of experiments on sixteen animals it was a regular finding that the column of bile in the manometer connected with the lower common duct had to be raised to a level 100 to 120 mm. above it before flow started. This always ceased when the column had fallen to the 80 or 90 mm. level. The values were obtained between 4 and 12 hours after feedings.

The Increase in the Resistance to the Flow of Bile into the Intestine during Fasting.

Fasting for 24 to 72 hours results in a great increase in the resistance offered at the lower portion of the common duct to the flow of bile

into the intestine. This finding was regularly obtained on twenty different occasions, in ten animals, that is to say in all of the tests made under the conditions. Whereas the pressure exerted by a column of bile 100 to 120 mm. in height usually sufficed to cause flow through the ampulla of Vater when the test was carried out 4 to 12 hours after a feeding, it was often necessary to raise the column to a height of 200 to 250 mm., to obtain this result in the fasting dog.

In relation to this phenomenon the findings of Bruno[2,3] and Rost[20] have a suggestive interest. They observed unanesthetized dogs with Pavlov biliary fistulas and state that during fasting periods bile emerged from the ampulla only in small amounts and at long intervals of $1\frac{1}{2}$ to $2\frac{1}{4}$ hours.

Type experiments follow.

Dog 3, Text-Fig. 1.—At the time when the experiments were begun, 83 days after intubation, the animal was healthy and active. The resistance offered to the flow of bile through the lower common duct was tested 2 hours after a feeding by bringing the manometer and reservoir filled with the animal's own sterile bile into connection with the tube leading to the lower common duct.

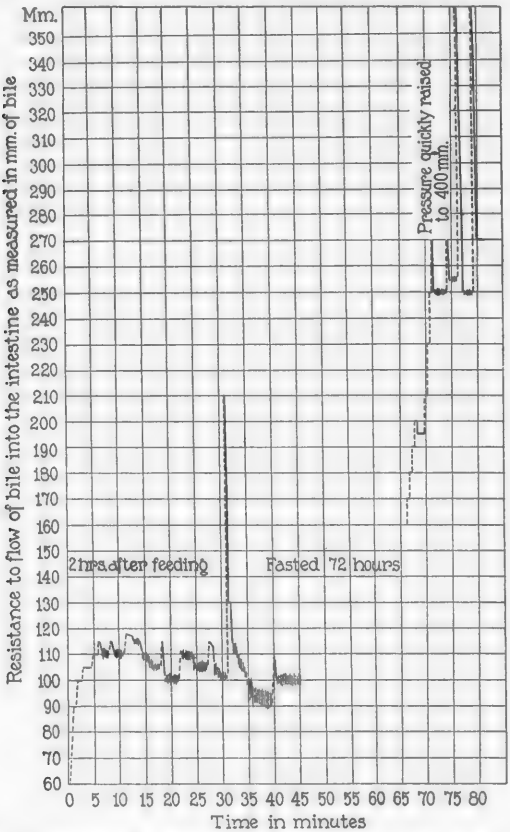
In Text-fig. 1 and in the others to follow, the dotted lines indicate an artificial raising or lowering of the level of bile in the manometer, the continuous lines indicate changes in the pressure level consequent on physiological changes within the animal influencing the resistance to flow. Wherever a sinking of the bile column is depicted by a continuous line it is due to the flow of bile out of the manometer, through the lower common duct, into the intestine. As the figure shows, for Dog 3 the column of bile in the manometer was gradually raised and frequently allowed to remain, unaltered by the observer, during 1 minute or more. When it had been raised by him to 115 mm. spontaneous fallings of the column occurred, to the level of about 100 mm. After the tests had continued over half an hour, the column of bile in the manometer was abruptly raised to 200 mm. It fell almost as promptly to 100 and then more gradually to 90 mm. When the column of bile was now raised to 110 mm. there was a fall to 100 mm. as in previous tests of the sort.

Food was withheld for 72 hours, and after this the tests repeated. As the second portion of Text-fig. 1 shows, the column of bile in the manometer was raised gradually, with frequent pauses to give time for readjustment to take place on the part of the animal. Only when a pressure of 270 mm. had been reached did any flow into the intestine take place and this was checked when the pressure had fallen to 250 mm. Raising the column again to 270 mm. caused a drop to the 255 mm. level. Two attempts to cause lessening of the resistance to the flow of bile into the intestine by increasing the pressure considerably failed of

their object. Even when the bile column had been raised to the height of 400 mm., it fell back only to the 250 mm. level.

Dog 4, Text-fig. 2.—36 days after intubation the animal was healthy and active, following a fast of 48 hours.

11.00 a.m. Flow did not start until the column of bile in the manometer con-

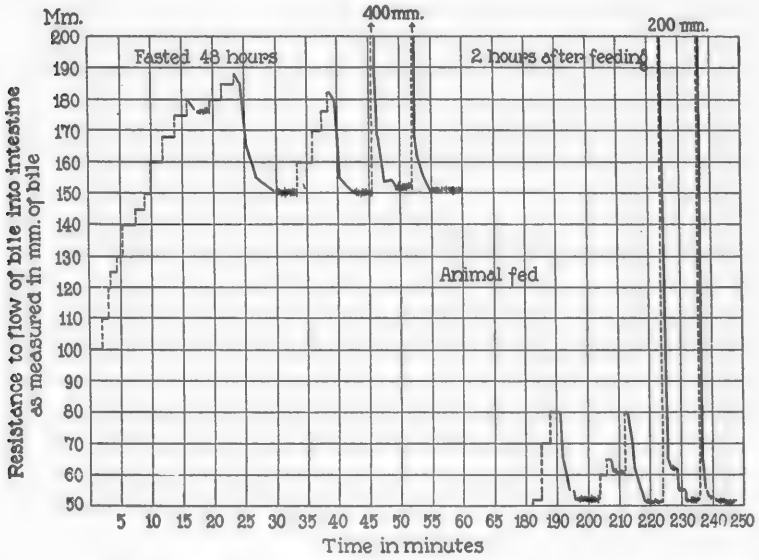


TEXT-FIG 1. Influence of fasting on the resistance offered to the flow of bile into the intestine. See text.

nected with the lower common duct was raised to a level 180 to 190 mm. above it. Flow ceased when this had fallen to 150 mm. Twice thereafter the bile column was raised to 400 mm. but each time bile flow ceased when the pressure had fallen to about the 150 mm. level.

12.00 noon. The dog was given food. 2 hours later a flow of bile into the intestine occurred repeatedly at the pressure exerted by an 80 mm. column of bile, and did not cease until the pressure had fallen almost to 50 mm. When the column of fluid in the manometer was raised to 200 mm. a fall followed and bile did not cease to pass into the intestine until the 50 mm. level had been reached.

Dog 5.—48 days after intubation the animal was healthy and active. 6 hours after feeding, 150 mm. of bile, in the manometer connected with the lower common duct, was sufficient to cause bile flow through the ampulla. This ceased when the level fell to 135 mm.



TEXT-FIG. 2. Effects of fasting and of feeding on the resistance offered to the flow of bile into the intestine.

The dog was then fasted and 2 days later the tests repeated. A 240 mm. column of bile was needed to start flow. This ceased at the 200 mm. level.

Immediate Decrease in the Resistance to the Passage of Bile into the Intestine at the Perception of Food.

Increased resistance to the passage of bile into the intestine during a fast was a constant finding in all the experiments made upon ten different animals. It now became a matter of interest to study the

direct influence of feedings. Accordingly food was proffered to a fasting dog while a manometer was connected with the tube leading to the lower common duct. The recorded pressure had shown a great resistance to the passage of bile into the intestine, as always during a fast. At the mere perception of the food, before any had been given the animal, the column of bile suddenly dropped. This experiment was tried again and again in ten different animals and in over 75 per cent of the trials the result was the same, that is to say there was a relaxation of the musculature about the lower end of the common duct, due, most obviously, to a "psychic reflex." In these instances the actual act of eating often brought about a further relaxation as evidenced by a still greater drop of the bile column (Text-figs. 3 to 5). In all instances, including those in which no drop occurred at the perception of food, the act of eating regularly caused a sudden decrease in the resistance to the passage of bile into the intestine as shown by a drop of the column of bile in the manometer. Soon after this a somewhat increased resistance made its appearance. This will be discussed below.

Usually but two or three experiments demonstrative of the psychic effect could be performed with one animal, because the mere act of placing it on a table for connection with the manometers brought about a conditioned reflex—the animal sniffed expectantly, became excited, and by the time the tubes could be connected tothe manometer the change in resistance to bile flow which it was desired to study had already occurred,—a low column of bile now sufficed to cause flow through the ampulla.

What appears to be a phenomenon of similar nature was recorded years ago by Bruno[2] in Pavlov's laboratory.

Bruno worked with a dog in which a Pavlov biliary[4] fistula had been made,—the ampulla of Vater surrounded by a good portion of duodenum had been transplanted into the anterior abdominal wall and the defect in the duodenum closed. On six occasions the animal was shown food but not allowed to eat, and on two of these occasions bile was seen to issue from the ampulla in amounts of 1.7 and 1.9 cc. respectively, though ordinarily the secretion did not appear until 15 to 45 minutes after the taking of food. Bruno also records the observation of Pavlov that gastric juice collected from fasting dogs during periods of "fictitious feedings" frequently contained appreciable amounts of bile. These instances of apparent sphincter relaxation are classed by Bruno among "psychophysiologic" reflexes.

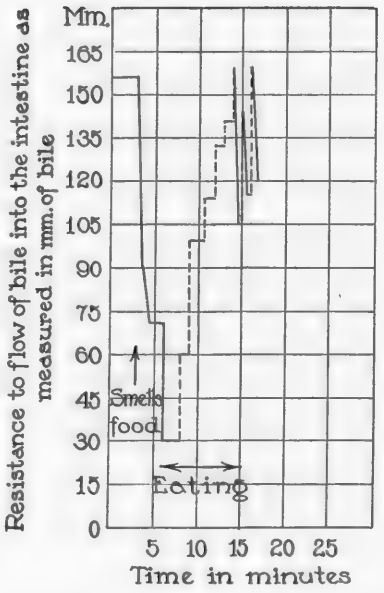
The phenomenon as we have observed it is represented graphically in Text-figs. 3 to 5. In Text-figs. 3 and 5 one can note as well the further lessening in resistance to the passage of bile into the duodenum when the animal began to eat.

Dog 5, Text-Fig. 3.—55 days after operation the dog was healthy and active. Following a fast of 72 hours the resistance to bile flow was such as to uphold a 155 mm. column of bile in the manometer attached to the tube leading to the lower common duct. The animal was then shown food and at the first perception of it the column dropped immediately to 90 mm. and in 1½ minutes to 70 mm. Eating was then allowed and at the first swallows the pressure fell at once still further, to 30 mm. The animal continued to eat. The column was raised within a period of 2 minutes to 60 mm. by allowing bile to flow into the manometer from the reservoir. No flow through the ampulla now occurred. The column was then raised to various levels as shown in the chart and allowed to remain 1 or 2 minutes at each. It did not fall until it reached the 160 mm. level. Following the initial decrease in the resistance offered to the flow of bile there had occurred almost immediately an increased resistance to it.

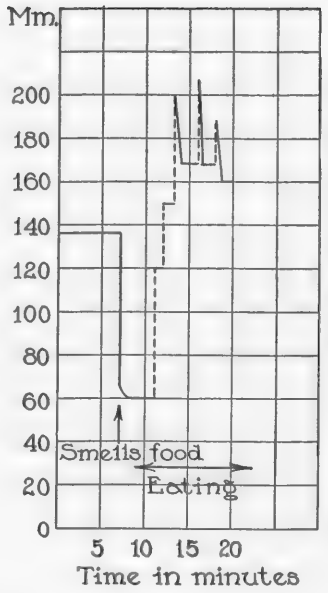
Another experiment on the same dog is represented in Text-fig. 4. It was performed 10 days later and after a fast of 48 hours. The result was similar but not as marked. The level of bile in the manometer connected to the lower common duct, and standing at 135 mm. above the latter, dropped to 60 mm. at the first perception of the nearness of food. No further change occurred with the actual taking of food but 2 minutes later while the animal was still eating, the column of bile in the manometer was raised to various levels as shown in the chart. Within 6 minutes it had been raised to 200 mm. Only then did flow of bile through the ampulla occur.

Dog 9, Text-Fig. 5.—35 days after operation the dog was healthy and active. Following a fast of 72 hours the resistance to the flow of bile into the intestine was tested as shown on the chart and found capable of supporting a 240 mm. column of bile. Food was then offered to the animal and immediately at the perception of it and before any had been eaten the bile column in the manometer dropped to 175 mm. and a minute later to 135 mm. Following the first swallows of food it fell further to 80 mm. After a minute of eating the column was raised by allowing bile to flow into the manometer from the sterile reservoir and after several such tests which disclosed a gradually increasing pressure the resistance was found to be high enough to hold back a column of bile 400 mm. in height.

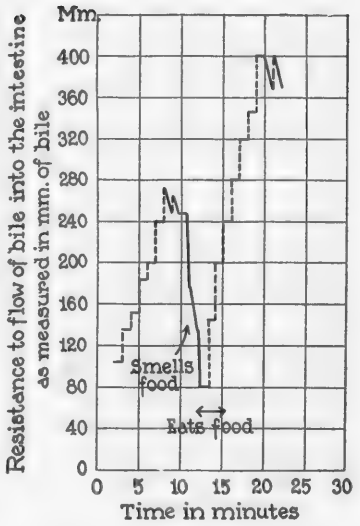
The phenomenon was also demonstrated by measuring the amount of fluid passing through the ampulla of Vater at a constant pressure, using for such determinations a type of "flow" manometer. Any decrease in the resistance offered to the passage of fluid into the intestine



TEXT–FIG. 3.



TEXT–FIG. 4.

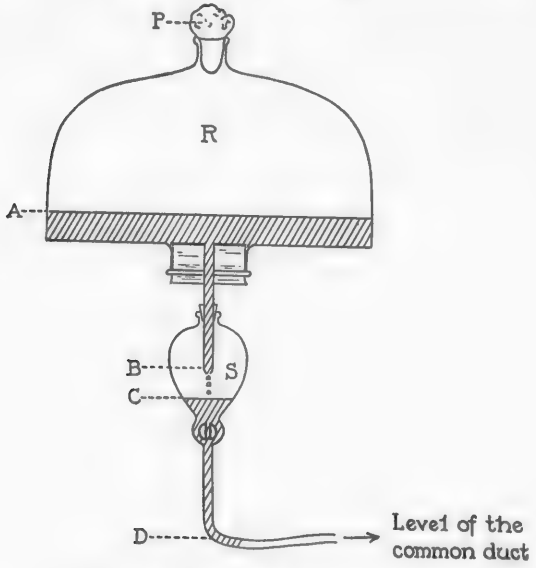


TEXT–FIG. 5.

TEXT-FIGS. 3 to 5. Influence of the sight of food and feeding upon the resistance offered to the flow of bile into the intestine.

resulted in a sudden increase in the rate at which the fluid flowed through the apparatus into the ducts.

The manometer used for this purpose is represented schematically in Text-fig. 6. A reservoir (*R*) was connected with a tube to an ordinary separatory funnel (*S*) which could be attached in turn to the tube leading to the lower common duct. The apparatus was sterilized by boiling or autoclaving. With all joints air-tight, the reservoir alone was open to the air but protected from contamination with a sterile cotton plug (*P*). By adding the heights of the two columns of fluid *A* to *B* plus *C* to *D* the pressure exerted by them could be estimated; and this

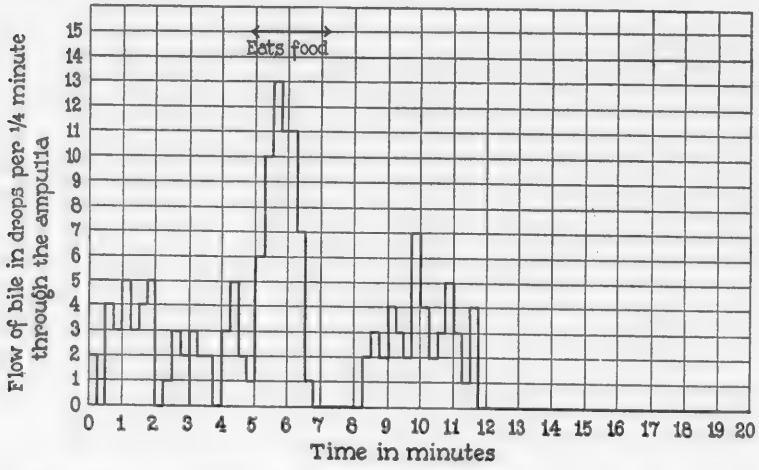


TEXT-FIG. 6. Diagram of the flow manometer.

could be varied at will by raising or lowering the apparatus. The rate of bile flow through the ampulla was measured roughly by counting the drops issuing from the nozzle *B* in quarter-minute intervals. Since 3 to 6 cc. of bile entered the intestine during the observations the surface level in the large reservoir was not appreciably changed, for a flow of 10 cc. reduced this but 1 or 2 mm.

Dog 19, Text-Fig. 7.—The operation for intubation of the common duct under ether had been performed 10 days before and the dog was healthy and active. After a fast of 48 hours the "flow" manometer was connected to the tube leading to the lower common duct and the pressure exerted by the apparatus was so ad-

justed as to equal that of a column of bile 160 mm. high. There was a slight flow of about 50 drops (1.6 cc.) to the intestine before food was given, as the chart shows. But at the perception and taking of food there was a sudden and pronounced increase in this. Within 2 minutes three times as much fluid had entered the intestine as in the 2 minute period preceding the taking of food. Flow then ceased entirely. 1½ minutes later there was a second period of flow lasting 4 minutes. In all 105 drops, or about 3.5 cc., of bile flowed into the intestine in the 7 minutes following the taking of food.


Eats food
Flow of bile in drops per ¼ minute through the ampulla
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Time in minutes

TEXT-FIG. 7. Influence of food ingestion on the flow of bile into the intestine.

The Secondary Increase and Decrease in the Resistance to the Flow of Bile into the Intestine after Feeding.

Following the transient decrease in the resistance to the passage of bile through the lower common duct after taking food, as described in these experiments, we observed a prompt increase in it as has just been noted. This was found by later experiments to endure for a variable time, becoming gradually less and less. Within half an hour or more the "normal" resistance was approached, namely that capable of supporting a column of bile 100 to 120 mm. in height. The increased resistance shortly after feeding has been illustrated in the experiments described above (Text-figs. 3, 4, 5, and 7). Babkin[5] has reviewed the experiments with a Pavlov biliary fistula and has

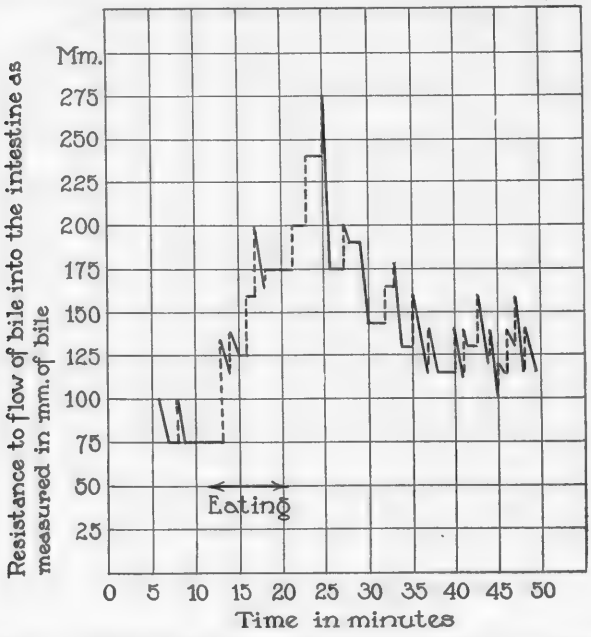
described a "latent period" between the giving of food and the first appearance of bile at the ampulla. The interval varied between 15 and 45 minutes depending upon the type of food given.

In our experiments the period of increased resistance in the lower common duct soon after feeding was of variable duration from 10 to 35 minutes. The degree of increase was often very great. In one case a 400 mm. column of bile was supported a few minutes after eating (Text-fig. 5). Eighteen feeding experiments in all were performed on ten dogs and the phenomenon was observed in every one. A type protocol will be given.

This experiment (see also Text-fig. 8) is chosen for description because it was carried out upon an animal fasted and then fed, several times previously, while the manometers were connected to the tube leading to the lower common duct. Through the experience of the repeated tests the animal had developed a conditioned reflex which resulted in the passage of bile into the intestine at a low pressure, but in spite of this a secondary increase in the resistance offered to passage regularly occurred after food was taken, and it became necessary to raise the column of bile in the manometer to a level of 275 mm. before fluid flowed into the intestine. Soon after this, however, relaxation of the musculature about the lower common duct began, as shown by the passage of bile through the ampulla at lower pressures.

Dog 9, Text-Fig. 8.—45 days after intubation the animal was healthy and active. It had been fed many times while manometer measurements upon it were under way. No food had been allowed for 48 hours previous to the present test. The animal became restless and sniffed about expectantly when placed on the table in order that the manometer might be connected to the tube leading to the lower common duct. It was not surprising, therefore, to find when this had been done, that a column of bile only 100 mm. in height was sufficient to cause a flow of bile through the ampulla, as the chart shows. The animal was shown food and permitted to eat. Within 2 minutes a 125 mm. column of bile was supported and in 10 minutes one of 240 mm. although a much lower column had caused flow into the intestine a few minutes before. Only after 20 minutes did the resistance decrease so that bile passed into the intestine at a lower pressure head, 175 mm. In half an hour the resistance had lessened to that originally noted, one supporting a column of about 100 mm. of bile; but even then it fluctuated. At one moment flow of bile into the intestine occurred at a pressure of 120 mm., and a little later only at one of 160 mm. Tests were made at short

intervals, the results of each being represented on the chart. The broken lines indicate artificial raisings of the pressure, the solid lines movements of the column of bile in the manometer in response to changes in the resistance.



TEXT-FIG. 8. The secondary increased resistance offered to the flow of bile into the intestine, following ingestion of food, and its gradual lessening, as shown by variations in the height of a column of bile in the manometer.

The Effect of the Ingestion of Weak Acid and Alkali upon the Resistance Offered to the Passage of Bile into the Intestine.

The reaction of the gastric contents may conceivably influence the resistance to the flow of bile into the intestine. For there is an immediate lessening in the resistance upon the taking of food, and a gradual increase in the half-hour or hour following, during which the reaction of the gastric contents is presumably becoming more acid. Since food may at this time be passing through the pylorus, a possible mechanical effect of peristalsis may in addition be invoked to explain the findings.

Fasting dogs were fed weakly acidified and alkalized solutions and the resistance offered to the passage of bile through the ampulla was estimated at such times. Very recently while these experiments were under way Cole[21] has reported observations of the sort on the anesthetized dog. He introduced acid or alkali into the stomach and observed an immediate decrease or increase respectively in the resistance to bile flow into the intestine as measured by the height of a column of fluid supported in a manometer tied in the common duct. We have noted the same relationship, though owing to the impossibility of controlling precisely the reaction of the gastric contents in the unanesthetized animal, our findings were not as constant as Cole's.

Seven experiments were performed in four animals appropriately intubated. Various amounts of N/10 HCl or 5 per cent $NaHCO_3$ were given to the fasting animals by stomach tube. A type experiment follows.

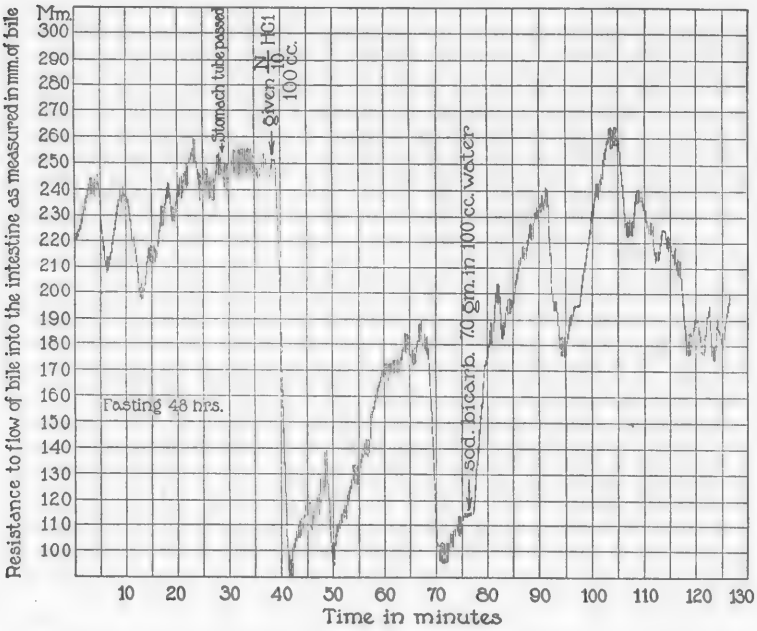
Dog 9, Text-Fig. 9.—18 days after an "altercursive intubation," the animal was healthy and active. No food had been given for the 48 hours previous to the experiment. An empty manometer was substituted for the balloon and, with the secretion of bile by the liver, a column of it 220 mm. high collected as a result of the resistance to passage of the secretion into the intestine. The findings after this level had been attained are charted in Text-fig. 9. They were recorded by the cooperation of two workers, one constantly observing and calling out the level of the bile column in the manometer, the other, who held a stop-watch, plotting as ordinates on graph paper the reported level with minutes as the abscissæ. The text-figure is the graphic representation of the findings obtained in this way, not a kymographic tracing, which it superficially resembles. The slight vertical fluctuations in the level of the bile column which will there be noted were due to respiratory movements. As the chart shows, a column of bile about 250 mm. in height could be maintained in the manometer. Slight decreases in the resistance occurred but the column did not fall below the 200 mm. level.

It has been estimated[25] that the acidity of gastric juice is equivalent to or slightly stronger than that of N/10 hydrochloric acid. Accordingly the animal was now given 100 cc. of N/10 HCl by a stomach tube already *in situ*. The tube had been passed 10 minutes previously without affecting the level of bile in the manometer. Practically at once, upon giving the acid, a drop of the bile column in the manometer to 90 mm. occurred. 10 minutes later when the pressure ex-

[25] Menten, cited by Mathews, A. P., Physiological chemistry, New York, 4th edition, 1925, 357.

pressive of the resistance had risen but only to 130 to 140 mm. another drop took place and again, 20 minutes later, when the manometer showed that the pressure in the biliary tract had risen to the equivalent of the weight of a column of bile 180 to 190 mm. in height, it once more, without obvious cause, fell rapidly to 100 mm.

Now 7.0 gm. of sodium bicarbonate in 100 cc. water was given. 15 minutes later the bile that was being secreted had raised the pressure in the biliary tract



TEXT-FIG. 9. Influence of the ingestion of acid and alkali upon the resistance offered to the flow of bile into the intestine.

to 230 mm., and 10 minutes later to 260 mm., when there once more occurred a flow into the intestine with a fall in pressure.

Control feedings of water elicited no changes in the resistance offered to the flow of bile into the intestine.

Experiments like this one, while not conclusive in themselves, point to the occurrence of changes in the resistance to the flow of bile through the common duct referable to alterations in the reaction of the gastric contents.

DISCUSSION.

The work here reported is the first of this nature, so far as we are aware, that has been done upon animals under practically normal conditions.[26] Since the approach to the normal is of prime importance, procedures rendering the musculature of the lower portion of the common duct visible have been avoided. Under the circumstances of the experiments described it has been possible to measure the resistance offered to bile flow through the ampulla at any time desired. We have used this measurement as a criterion expressive of the conditions under which bile makes its escape into the intestines. No attempt has been made to differentiate experimentally the activities of the duodenal musculature about the lower common duct as apart from that of the sphincter of Oddi. Recently Carlson[22] and Burget[23] have stressed the possible importance of the duodenal musculature in the retention or escape of bile. It undoubtedly plays a rôle especially during active peristalsis. More will be said of this below.

If the control of bile flow through the ampulla is due to the sphincter it might be objected that the operative interference incident to intubation of the common duct would probably destroy or at best interfere with nerve filaments passing to and from the muscle of Oddi. Since our findings were positive ones, the sphincter retaining its power of relaxation and contraction, such disturbances cannot have been very serious. Either the nerves affected play an unessential part in the function of the sphincter, or they had reestablished their normal connections.

Whatever be the explanation of our findings, they show definitely that the escape of bile into the duodenum is under a definite control and is markedly influenced by varying the physiological conditions.

In these experiments there was a remarkable constancy of the findings. In the normal unanesthetized animal recently fed the resistance to the passage of bile through the ampulla supports a column of bile or physiologic saline solution 100 to 120 mm. in height, a finding

[26] Since this was written, J. C. Potter and F. C. Mann have published in the *Am. J. Med. Sc.*, 1926, clxxi, 202, studies on the "Pressure changes in the biliary tract" in unanesthetized dogs, also under practically normal conditions. We regret that the appearance of this most interesting paper occurred too late to permit of discussion of their findings herein.

which agrees well with many of the observations of others made under ether.[8] In the fasting animal this resistance is always without exception much increased. However, at sight and smell of food it usually decreases and invariably does so at once upon eating, and, as our experiments show, permits an increased flow of fluid through the ampulla, at constant pressure. This marked but transient relaxation is followed almost as soon as food reaches the stomach by an increased resistance to the flow of bile which endures for about half an hour. Experiments with acid and alkali feeding show that the sequence of events just described with the exception of that due to the psychic stimulus may be explainable by changes in the gastric acidity after the taking and digestion of food. That this may not be the only factor engaged, and probably is not, is well recognized by us.

Whether the intermittent ejection of acid chyme through the pylorus stimulates relaxation of the musculature about the lower common duct cannot be determined from our observations. In favor of such a view is the finding that the resistance offered to bile flow through the ampulla during gastric digestion is a fluctuating one (see Text-fig. 8), but almost all our further evidence argues against it. For example, the lessening in the resistance to the passage of bile into the intestine which occurs when food is shown the animal takes place so promptly and rapidly that it must certainly be the result of a reflex. The same may be said of the rapid relaxation following administrations of acid to the fasting animal (Text-fig. 9). In the fluctuation of the resistance to the flow of bile through the ampulla during gastric digestion the duodenal musculature may well play a part.

A consideration of the rôle of the gall bladder in the passage of bile into the intestine is essential to any discussion of the physiology of bile flow. This will be taken up in a succeeding paper.

SUMMARY.

Under specially controlled conditions in the healthy unanesthetized dog we have measured the resistance offered to the flow of bile through the lower common duct.

The average resistance 4 to 12 hours after a feeding was found to correspond to the pressure of a column of bile 100 to 120 mm. in height.

After a 24 to 72 hour fast the resistance was such as to support a much higher column of bile, one of 300 mm. at times. The exhibition of food to the fasting animal usually precipitated a reflex lessening in the resistance to the flow of bile to the intestine, the actual taking of food always brought it about. This was transient and was soon followed by a period of increased resistance lasting 10 to 30 minutes after food had entered the stomach. There succeeded a drop in resistance which was gradual and fluctuating.

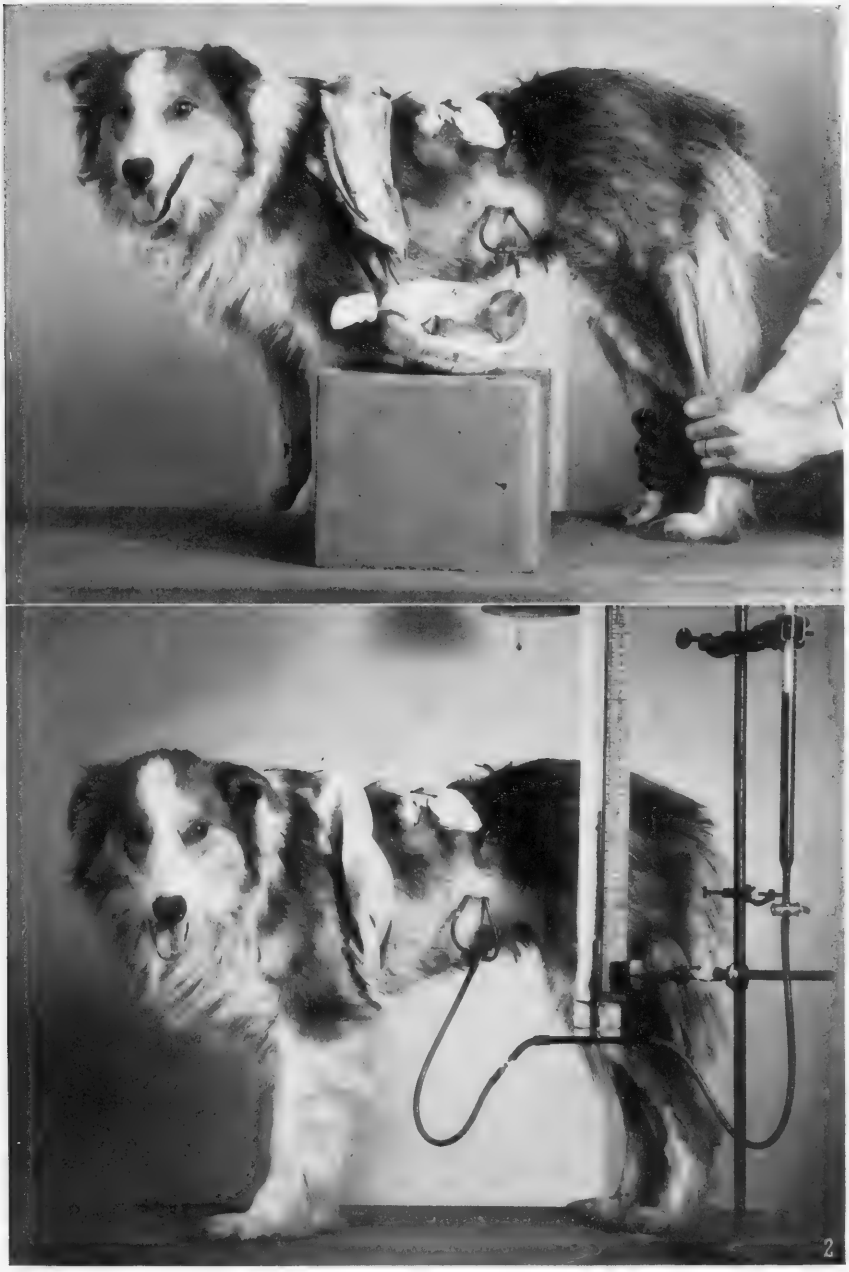
We have observed an increase in the resistance to the flow of bile into the intestine after alkali has entered the stomach, and a decrease after acid has been administered.

We attribute the sudden changes chiefly to the activity of the sphincter of Oddi.

EXPLANATION OF PLATE 5.

FIG. 1. Animal with an "altercursive intubation." For description see text.

FIG. 2. Manometer connected with the collecting tube of an "altercursive intubation." For description see text.



(Elman and McMaster: Resistance to bile flow to intestine.)

ON THE EXPULSION OF BILE BY THE GALL BLADDER; AND A RECIPROCAL RELATIONSHIP WITH THE SPHINCTER ACTIVITY.

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In the present paper we shall offer proof that the gall bladder discharges bile on physiological occasion, through the agency of forceful contractions. The phenomenon has been encountered in healthy, unanesthetized animals with the biliary passages permanently intubated in such a way[1–3] that the pressure exerted by the vesica fellea and the resistance to the flow of bile into the gut could be studied simultaneously and under controlled conditions. The existence of a relationship between the activity of the gall bladder and that of the musculature about the lower common duct, as expressed in alterations of the resistance just mentioned, has been demonstrated by the method.

The most diverse views of gall bladder activity and function have been proposed in the past, and they all still find adherents. Some workers hold the gall bladder to be an active, contractile viscus,[4–8] capable of expelling its contents. In contrast to such an opinion Sweet,[9]—who reasons from the anatomic structure of the human viscus and cystic duct,—believes that nothing entering the gall bladder through the duct ever leaves it by the same route but is sooner or later resorbed. The midground between these positions is held by many ob-

[1] Elman, R., and McMaster, P. D., *J. Exp. Med.*, 1926, xliv, 151.
[2] McMaster, P. D., and Elman, R., *J. Exp. Med.*, 1925, xli, 513.
[3] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1923, xxxvii, 11.
[4] Doyon, M., *Arch. physiol. norm. et path.*, 1893, v, series 5, 678, 710.
[5] Bainbridge, F. A., and Dale, H. H., *J. Physiol.*, 1905, xxxiii, 138.
[6] Okada, S., *J. Physiol.*, 1915–16, l, 42.
[7] Judd, E. S., and Mann, F. C., *Surg., Gynec. and Obst.*, 1917, xxiv, 437.
[8] Mann, F. C., and Giordano, H. S., *Arch. Surg.*, 1923, vi, 1.
[9] Sweet, J., *Internat. Clin.*, 1924, i, 187.

servers. Winkelstein,[10] most recent among them, believes that the organ is purely passive, filling and emptying as result of extraneous alterations of the pressure brought to bear upon it, more especially in the course of respiration. That the gall bladder fills with bile is disputed by nobody.

Renewed interest in the problem of the contractility of the gall bladder has sprung from an application to it of the law of contrary innervation proposed by Meltzer.[11] He advanced an attractive hypothesis according to which, at each discharge of acid chyme from the stomach, the sphincter of Oddi relaxed and the gall bladder synchronously contracting, ejected a gush of bile into the duodenum. He noted in another connection that local applications of magnesium sulfate solutions to the duodenal mucosa cause relaxation of the muscles of the intestinal wall. The clinical application of this hypothesis and fact by Lyon[12] has resulted in many attempts at "non-surgical drainage of the gall bladder" with the accumulation of a great mass of conflicting evidence as to whether this object is ever accomplished. Since the literature has been reviewed by others[13-15] we need not enter into it here.

In a companion paper to the present one[1] it has been shown that an immediate decrease in the resistance offered to the passage of bile into the intestine takes place when an animal begins to eat, and usually indeed upon the mere perception of food. Conditions at such times are especially favorable to the escape of bile into the intestine. Does this occur in quantity, and what are the forces promoting it? Our first experiments have been directed to these points.

Technique.

A full description of the technique used in our studies has been given in the accompanying paper.[1] We have utilized healthy dogs in which some time previously there was installed, under ether anesthesia, the apparatus of an "altercursive intubation"[2] whereby bile flowing from the liver can either be collected under sterile conditions or turned back at will into the common duct to flow as usual through the ampulla of Vater into the duodenum. In order to study the influence of the gall bladder, the connections of the organ were left

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[10] Winkelstein, A., *J. Am. Med. Assn.*, 1923, lxxx, 1748.

[11] Meltzer, S. S., *Am. J. Med. Sc.*, 1917, cliii, 469.

[12] Lyon, B. B. V., *J. Am. Med. Assn.*, 1919, lxxiii, 980.

[13] Mann, F. C., *Physiol. Rev.*, 1924, iv, 251.

[14] Friedenwald, J., Martindale, J. W., and Kearney, F. X., *J. Med. Research*, 1922, ii, 349.

[15] Matsuo, I., *J. Am. Med. Assn.*, 1924, lxxxiii, 1289.

intact in a number of animals, with result that the cannula inserted into the upper part of the common duct formed the means of exit for both liver and gall bladder bile; while in others the cystic duct was sectioned and ligatured at the time of the intubation. In some cases the gall bladder influence was studied more directly by intubation of the cystic duct or of the duct which results from the union of this latter with the small tributary from the left central lobe of the liver. As an "altercursive intubation" of the common duct had also been carried out in these cases, they were the subject of what one may term a "triple intubation," the details of which will be described more fully below.

As in our many previous intubations of various sorts aseptic precautions were taken in the handling of the tubes. Only instances in which the bile and biliary tracts remained sterile will be considered in this paper. Cultures of the bile were taken at frequent intervals during the course of the observations and stained sediments from it, as obtained by centrifugation, were examined for bacteria. At autopsy the sterility of the bile was again ascertained by culture. Cultures from the liver tissue were also made on agar and in bouillon. Despite the most careful precautions the bile of many of our animals became infected owing to the constant handling of the tubes. All such instances were discarded as, too, were any suggestive of obstruction in the cannula-tube systems.

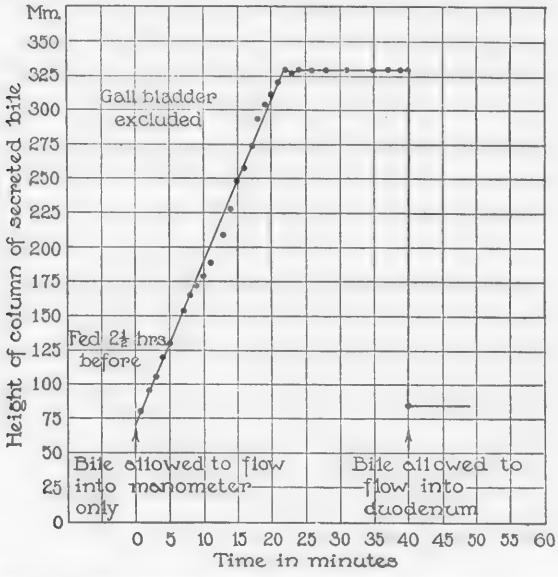
The animals were given a diet of bread wet with milk, and lean meat, 450 gm. of the latter to each kilo of the bread and milk. They were fed daily in the morning, and allowed to eat until satiated. The time when the animal was last fed proved to be an important factor in the results and careful record was made of it.

To determine the pressure exerted upon the contents of the gall bladder and bile ducts, and to measure resistance to the flow of bile into the intestine, the rubber tubes leading from and to various regions of the biliary tract were repeatedly brought into connection with sterile manometers previously filled with the animal's own sterile bile as collected some time previously. The changes in the level of the columns of bile in the manometers were recorded by the cooperation of two or four observers, according to the number of manometers to be observed. The watchers at each of these instruments read off all the alterations in levels of the bile columns in the manometers as they occurred, while the other workers, who held stop-watches, plotted, on graph paper, the pressures as ordinates against time in minutes as abscissæ. Text-figs. 1, 2, 5, and 6 represent these graphs as obtained.

Secretion Pressure of the Bile.

Before the action of the gall bladder can be intelligently discussed it is necessary to consider the cause of the pressure existing within the biliary tract. Of the forces producing it, that of bile secretion by the liver is chief. This has been well studied, notably by Herring

and Simpson,[16] who found in dogs under chloroform anesthesia with the gall bladder excluded, that the bile filled a manometer, to an average maximum of 300 mm. when obstruction was produced. The animals were fed 6 hours before the experiment. The time required for the secretion to reach the high level was variable, from 1 or 2 to often as much as 4 hours. Mitchel and Stifel[17] found in



TEXT-FIG. 1. The maximum secretion pressure of bile and its rate of formation in the presence of a pressure obstacle. The dots record the actual observations on an animal. It will be seen that the rate of secretion was unaffected by the increasing pressure obstacle.

the bile ducts of cats anesthetized after total biliary obstruction of 2 to 6 days duration a pressure equivalent to 250 to 300 mm. of water.

It seemed advisable to determine the pressure developing on

[16] Herring, P. T., and Simpson, S., *Proc. Roy. Soc. London, Series B*, 1907, lxxix, 517.

[17] Mitchel, W. T., and Stifel, R. E., *Bull. Johns Hopkins Hosp.*, 1916, xxvii, 78.

obstruction in our unanesthetized animals. Accordingly, many days or weeks after intubation, the liver bile of dogs with gall bladder excluded by severance of the cystic duct was allowed to flow directly into a vertical manometer tube from which there was no escape except by way of the opening at the top. The fluid rose to a height slightly greater than that noted by Herring and Simpson in anesthetized animals, that is to say to about 320 mm. on the average, the figures for the individual animals, two of which were tested a second time, being as follows: 302,* 305, 308,* 314, 318, 321, 324, 326, 326,† 330, 333,† 335.[18] The time required to reach the maximum point was much shorter than in the experience of these observers, being only 15 to 30 minutes. No relation was evident between the size of the animal and the pressure obtained.

In Text-fig. 1 is charted an instance typical of all. The animal yielding it had undergone an "altercursive intubation" 2 months before and was healthy and active. $2\frac{1}{2}$ hours before the experiment was begun food was given. The tube draining the liver bile, which up to that time had been delivering the fluid to the intestine by way of the rubber detour, was then brought into connection with a vertical glass manometer of 3 mm. bore. As the liver secreted bile the column rose rapidly to a level 330 mm. above that of the duct-cannula junction. It will be noted that despite the progressive pressure obstacle the rate of secretion remained constant, the bile column rising regularly, so that the readings of its height plotted against the time formed a straight line. The finding was obtained in all such experiments, twelve in number in ten different animals. The formation of bile, then, was as rapid against high pressures as low, until a maximum point was reached at which secretion suddenly ceased in the face of the high pressure.

When bile was collected at zero pressure for the brief periods necessitated by the experiments the same uniformity was noted in the rate of secretion from minute to minute. More will be said of this below.

[18] * and † indicate that the figures were obtained from the same animals upon different occasions.

The Effect of Food on the Flow of Bile in Animals with Gall Bladder Excluded.

Although changes in the pressure exerted against bile secretion do not affect its rate of formation, the giving of food does. This latter fact has long been a truism of physiology.[19,20] Yet as a preamble to further observations, it has been necessary to study the phenomenon carefully in our dogs.

In eight animals with ligated and sectioned cystic duct, the tube draining liver bile was connected to a sterile graduate and the amounts of bile secreted during 5 minute periods were recorded. While the bile was being collected the animals were allowed to eat the bread, milk, and meat mixture for 2½ to 3 minutes. No change in the rate of secretion was noted during the first 5 to 15 minutes. Then a gradual progressive increase appeared, as depicted in the lower portions of Text-figs. 5 and 6. The stimulus was short lived, the amount of bile secreted returning to "normal" in about 45 minutes. The bile was not markedly changed in its gross appearance at any time—it was still limpid, and a rather light brown in color—but it usually was found on analysis to contain less pigment per unit of volume than before the feeding, in conformity with previous knowledge.[21,22] When, however, the experiment was repeated in animals with the gall bladder connections undisturbed, the bile obtained after feeding was very different from that voided previously. Before this latter finding can be discussed in detail we must digress to consider certain pressure relations existing within the biliary tract.

The Filling of the Gall Bladder and Retention of Its Contents.

In an earlier paper from this laboratory[23] it was shown that the pressure exerted by a column of bile 60 to 70 mm. high connected with the common duct is sufficient to cause a flow of bile into the gall bladder of the anesthetized dog. In the unanesthetized animal the pressure within the viscus is generally about equal to that of a column

[19] Bruno, G. G., Dissertation, St. Petersburg, 1898; and Klodnizki, Dissertation, St. Petersburg, 1902; cited by Babkin, B. P., Die äussere Sekretion der Verdauungsdrüsen, Berlin, 1914, 344.

[20] Bruno, G. G., *Arch. sc. biol.*, 1899, vii, 87.

[21] McMaster, P. D., Broun, G. O., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 395.

[22] Dastre, A., *Arch. physiol. norm. et path.*, 1890, ii, series 5, 800.

[23] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 47.

of bile 100 mm. in height, as our experiments to be detailed below will show. The secretion pressure being much more than this, it is obvious that no force other than that resulting from the liver activity is needed to fill the gall bladder, provided bile does not escape into the intestine. However, in our companion paper[1] several factors have been recognized which may conceivably be responsible for such an escape of bile through the alterations they excite in the resistance offered to bile flow by the musculature about the lower portion of the common duct. This resistance in the normal animal, fed 4 to 12 hours previously, is sufficient to support a column of bile 100 to 120 mm. in height. In the fasting animal, however, it will support one 250 to 300 mm. high. Immediately after taking food and again later, during the process of digestion, the resistance is greatly lowered, and a bile column 50 to 80 mm. in height suffices to cause a flow of bile through the ampulla. From this it is evident that save at these latter times the bile, as secreted, backs up perforce into the gall bladder, this being the way of least resistance. Whether there is an aspirating effect on the organ due to the respiratory movements, as suggested by Winkelstein,[10] it is not necessary to consider, for the foregoing facts alone will suffice to explain the filling of the organ. Once within the gall bladder the bile undergoes rapid concentration.[23] This concentrating activity may well be more important for the retention of the gall bladder contents than the valve-like reduplications of the mucous lining of the cystic duct (Heister's valves). What part these may play remains to be determined as does that of the circular ring of muscle sometimes situated in the cystic duct.[24]

The Influence of the Gall Bladder on the Pressure of Bile in the Common Duct.

From the circumstance that the gall bladder receives and concentrates large amounts of bile, as well as because of the delay which its presence causes in the development of jaundice after obstruction of the common duct,[25] it might be inferred that the organ acts to

[24] Westphal, K., *Klin. Woch.*, 1924, iii, 1105.
[25] Afanassiew, M., *Z. klin. Med.*, 1883, vi, 281.

prevent the development of a high pressure within the duct system. And such is the case.

In six animals an "altercursive intubation" was done without interference with the gall bladder connections. In such instances the bile simply made the detour of the tubes on its way to the duodenum, and encountered at the lower end of the common duct the normal resistance to its passage into the gut. This, from our figures given in the previous paper, would be sufficient, except after the taking of food, to keep the gall bladder full.

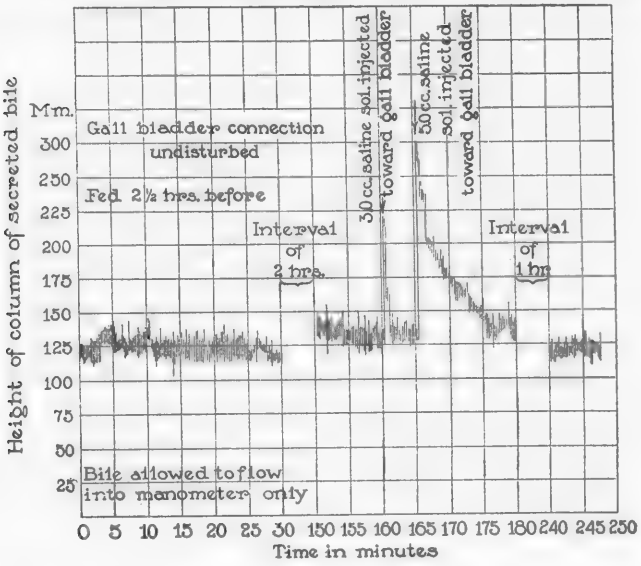
In nine experiments upon these animals with undisturbed gall bladders, we connected to a manometer the tube leading from the upper portion of the common duct, taking due care that no bile escaped from the latter during the process. The animals had been fed the usual meal of bread, milk, and lean meat several hours previously. During the succeeding several hours the column of bile never rose to more than 100 or 150 mm. above the level of the duct, that is to say it never more than approximately equalled the resistance which would normally have been exerted against the escape of bile into the intestine. In none of our experiments did the pressure within the common duct ever approach the maximum secretion pressure of the liver.

In Text-fig. 2 is plotted the result of such an experiment. In sharp contrast to this are the findings already described above (Text-fig. 1) when the gall bladder had been cut off from connection with the duct-manometer system.

Dog 12, 9½ kilos, Text-fig. 2. An "altercursive intubation" had been carried out 9 days previously, with the gall bladder connections left intact. The animal had been fed each morning and at the time of the observation was in the best of health. 2½ hours after feeding, the experiment was begun, the tube leading from the upper portion of the common duct being then connected with a manometer of about 3 mm. bore, one that is to say which required only 1 cc. of bile for a rise of 141 mm. in the column. As the chart shows, the pressure that developed and was maintained in the common duct during the next 4 hours equalled that of a column of bile about 125 mm. in height. There were frequently repeated, rapid but slight, incidental fluctuations due to the respiratory movements. Usually the column then rose or fell about 10 to 15 mm., occasionally on a deep breath 20 to 30 mm. The amplitude of the larger of these fluctuations and of certain more considerable yet still slight excursions of the

bile column in the manometer is shown in the text-figure which is typical of all such experiments. Only the readings of two brief periods each of half an hour and another of 10 minutes are recorded in the chart. As already stated the manometer remained connected and completely obstructing the common duct for over 4 hours.

From the failure of the bile column to rise far it was obvious that the gall bladder must be storing the bile almost as fast as it was formed. To test its ability in this connection, 3 cc. of 0.9 per cent sodium chloride solution was run



TEXT-FIG. 2. The pressure developing during the first few hours of total biliary obstruction in an animal with the gall bladder connections left undisturbed. Contrast with Text-fig. 1. The manometer was connected with the common duct for over 4 hours.

abruptly into the common duct through the tube connected with the manometer. The momentary increase in pressure caused by this disappeared almost at once. 5 minutes later 5 cc. more of the solution, injected into the common duct and toward the gall bladder, caused an increase in pressure sufficient to raise the bile column to 300 mm. in the manometer, that is to say, to almost the maximum pressure of total obstruction whether the gall bladder be in or out. There followed a rapid return of pressure to the previous level. Although the manometer remained in connection with the common duct for another hour there was still

no evidence of an increase in pressure within the biliary tract above 125 mm., the level "normal" for the animal; and the experiment was discontinued.

Unlike the "straight line curve" of increasing pressure upon obstruction, obtained in animals with the gall bladder excluded, as noted in Text-fig. 1, no rise in the curve occurred during the first 4 hours when the gall bladder was present.

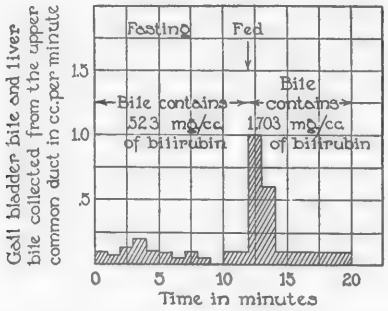
The Effect of Food on the Bile Yield of Animals with Gall Bladder Undisturbed.

In our dogs possessing intact gall bladders a decided difference was to be noted in the gross appearance of the bile collected before and after feeding, as already has been said. It may be recalled that the "altercursive intubation" in these animals permitted the bile simply to make a detour on the way to the gut. The normal resistance to the passage of bile into the intestine would be sufficient to divert the secretion to the gall bladder soon after a feeding.

The bile secreted by these animals when they had been fasting for 24 to 48 hours was allowed to drain freely into sterile graduates and then, after half an hour, food was given while the bile collection was continued. The secretion of these animals while fasting showed the characteristics of liver bile under such circumstances, that is to say it was more concentrated than in full fed animals; but immediately after the first swallows of food an abundant gush of far darker and more viscid bile suddenly flowed into the graduate, the darkness in color being due, as quantitation showed, to a high concentration of bilirubin. This experiment was performed twice with each of the six dogs with undisturbed gall bladder connections, and in all instances the same result was obtained. The phenomenon suggests, of course, a discharge of gall bladder contents. The finding (Text-fig. 3) should be compared with the delayed, and slow and gradual increase in the flow of bile observed after a feeding in animals with the gall bladder excluded from the collecting system (lower portions of Text-figs. 5 and 6). In instances of the latter sort in which liver bile alone is collected the food stimulus to secretion, far from causing the bile to become thicker and darker has effect to render it more abundant, more watery, and light in color,—a fact long known.

Specimen Protocols.

Dog 9, Text-fig. 3, weight 8½ kilos. 53 days prior to this experiment an "altercursive intubation" was carried out under ether. The gall bladder connection with the common duct was left undisturbed. During the long preliminary period the animal remained in excellent health, and bile was allowed to flow into the intestines as usual, by way of the altercursive detour. The dog was now fasted for 72 hours, after which the tube,—which drained not only the liver but the gall bladder,—was disconnected, and the amount of bile collected from it in each minute was recorded for half an hour. Food was then given

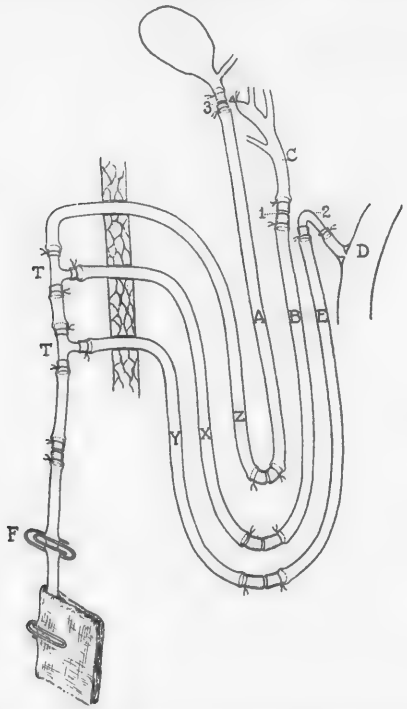


TEXT-FIG. 3. Food as a stimulus to the discharge of bile from the gall bladder. Bile was collected from the upper common duct draining both liver and gall bladder. The gall bladder connections had been left undisturbed. When food was given a large amount of viscid and highly pigmented bile was voided practically at once. Compare the finding with the gradually increasing output of bile from animals with the organ segregated (lower portions of Text-figs. 5 and 6). The differences in the amount and nature of the bile collected point to a discharge of bile from the gall bladder.

while the collection of bile was continued. As the chart indicates, only 1.1 cc. of bile had appeared during the 15 minutes before this. It was light in color, limpid, and contained 0.523 mg. of bilirubin per cc. Pigment determinations on it were carried out by a method described elsewhere.[21] Within 2 minutes after food was first taken a gush of dark viscid bile appeared (1.6 cc.) which contained upon analysis 1.703 mg. of pigment per cc.

Dog 11, weight 9¼ kilos. 18 days before this experiment an "altercursive intubation" had been done under ether. The gall bladder connection had been left undisturbed. Thereafter bile was allowed to flow to the intestines as usual. For the 72 hours previous to the experiment the animal was fasted, after which the bile from the tube draining liver and gall bladder was collected into a sterile graduate and the amounts recorded every 5 minutes.

In the first 10 minutes but 1.2 cc. of relatively light-colored limpid bile was obtained, though one not nearly so light as liver bile is in an animal which had not fasted. It contained 0.77 mg. of bilirubin per cc. The dog was then fed and in the next 10 minutes 10.2 cc. of a dark, viscid bile having a bilirubin content of 1.61 mg. per cc. flowed into the graduate.



TEXT-FIG. 4. Plan of the "triple intubation." Besides the tubes *XB* and *YE* leading from and to the common duct which constitute the so called "altercursive intubation" of previous papers, there is a third tube *ZA* communicating with the gall bladder. When the tube was clamped at *F* bile flow to the intestine went on as usual for weeks or months. There was merely a detour of the fluid to the outside.

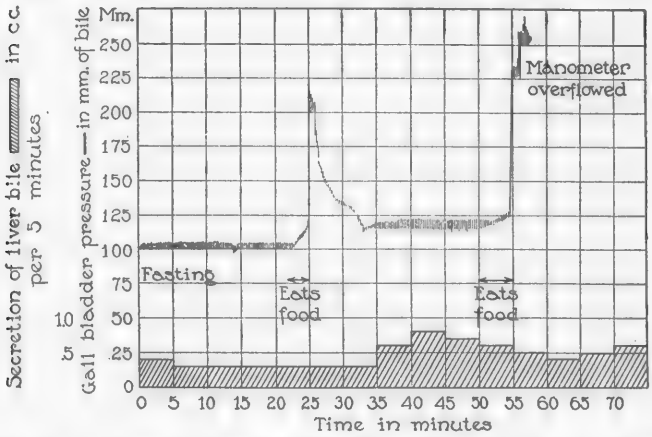
Proof that the Gall Bladder Expels Bile by Forceful Contraction.

The phenomena as described suggested a forceful discharge of bile from the gall bladder. In order to study them to advantage we

resorted to an accessory intubation, whereby a tube was connected directly with the organ in addition to the usual "altercursive intubation." This "triple intubation" already referred to is represented schematically in Text-fig. 4.

The third cannula, *3*, connected to the rubber tubes *A* and *Z* was placed either directly in the cystic duct or (as shown in the figure) just far enough below it to allow the tiny duct draining part of the left central lobe of the liver to enter above the point of intubation and thus supply bile directly to the gall bladder. The findings were similar by both methods of intubation.

The ends of the three tubes introduced into the abdomen were passed through



TEXT-FIG. 5. Pressure changes within the gall bladder after the taking of food. The upper curve shows the sudden increases in gall bladder pressure, immediately after eating. The lower columns show the amount of bile secreted by the liver in 5 minute periods.

separate orifices in the lateral body wall and joined outside by means of glass *T*-tubes and short rubber connections. All of these joints were protected with wrappings of phenolized gauze. When a clamp was set upon the drainage tube *F*, bile secreted by the liver into tubes *BX*, upon arriving outside the body, was free to flow in two directions—on into tubes *YE* and thence into the duodenum, or backward into tubes *ZA*, and thence to the gall bladder. This latter course would be taken when the physiological resistance to the passage of bile into the intestine was high. By disconnecting the tubes leading to the gall bladder and to the lower common duct and bringing them into connection with manometers it was possible to measure simultaneously the pressure conditions within the

gall bladder, the resistance to the passage of bile into the intestine, and the amount of liver bile secreted.

With five dogs triply intubated as described we have repeatedly observed upon the taking of food abrupt pressure increases in the column of bile in a manometer connected with the gall bladder,—clear evidence that the organ contracts. The data of one such instance are recorded in Text-fig. 5.

Dog 15, weight 11 kilos, Text-fig. 5. 10 days previous to the experiment a "triple intubation" had been done, with insertion of the third cannula directly in the cystic duct. The animal remained healthy and active. After a fast for 48 hours just prior to the experiment a manometer was connected to the tube leading to the gall bladder, while the tube collecting liver bile was allowed to drain into a sterile graduate. In the text-figure the amount of liver bile secreted in 5 minute periods is plotted in cross-hatched columns. During the first 20 minutes less than 0.5 cc. was obtained in each period and the column of bile in the manometer connected with the gall bladder remained constantly at the 100 mm. level, showing slight fluctuations referable to the respiratory movements.

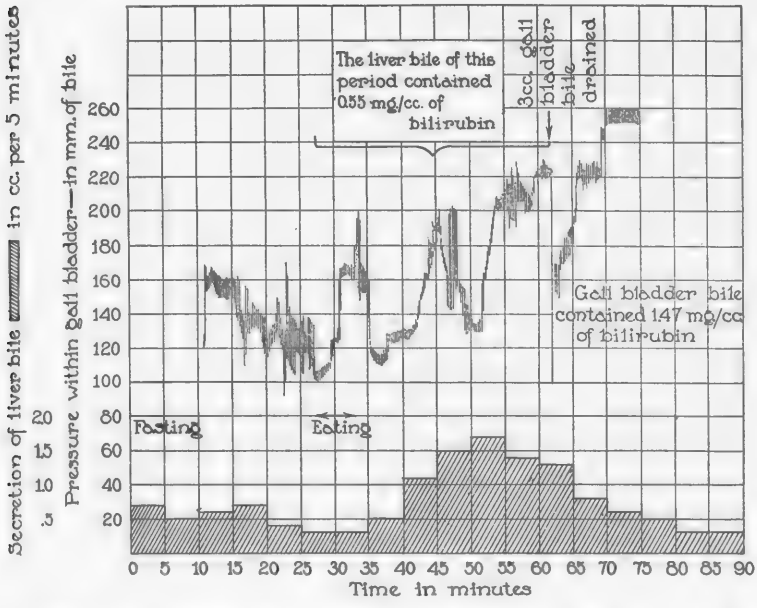
Food was then offered to the animal and it was allowed to eat for 2½ minutes, consuming in this time about 150 gm. Almost at once the bile column in the manometer, registering pressure changes within the gall bladder, rose to over 200 mm. and it returned again slowly toward its previous level in the course of the next 8 minutes reaching 115 mm. and there remaining. In the meantime food was removed. The column of bile in the manometer remained level until, 17 minutes later, food was again offered. Soon after eating the bile rose above the 250 mm. level, overflowed the manometer, and the experiment was discontinued. Through all this the animal stood quietly making no movements of its body. There is to be noted in the chart a gradual increase in the amount of liver bile secreted after the taking of food. Its flow was constant and continuous. No gush of fluid occurred from the cannula yielding liver bile, such as would suggest a generalized increase in the intraabdominal pressure, and of course no bile could reach the gall bladder from the liver because the tube leading to the viscus had been attached to the manometer. 4 days later while the animal was in excellent condition, it was killed with chloroform. At autopsy all the bile ducts were found normal in appearance and the cannulas patent.

The following instance shows the same phenomenon and its rhythmic recurrence in the absence of any further ingestion of food.

Dog 16, weight 9 kilos, Text-fig. 6. Under ether anesthesia a "triple intubation" was performed, and the three tubes inserted precisely as represented in

Text-fig. 4. It will be seen that a small duct from the left central lobe of the liver opened into the cystic duct above the point of intubation. Recovery from the operation was prompt and the animal continued active and healthy.

9 days after operation, and following a fast of 48 hours, the tube connecting with the gall bladder was joined to a manometer. The tube collecting liver bile was allowed to drain freely into a sterile graduate and the amount of bile received was recorded at 5 minute intervals for half an hour. A little food was then given, the animal eating about 150 gm. of the bread, milk, and meat mixture in 5 minutes.


Secretion of liver bile in cc. per 5 minutes
Pressure within gall bladder—in mm. of bile
The liver bile of this period contained 0.55 mg/cc. of bilirubin
3cc. gall bladder bile drained
Gall bladder bile contained 1.47 mg/cc of bilirubin
Fasting
Eating
Time in minutes

TEXT-FIG. 6. Pressure changes within the gall bladder after the taking of food. The upper curve shows four rhythmic increases and decreases in pressure each of 5 minutes duration or more. The cross-hatched columns record the quantities of liver bile voided. Feeding stimulated secretion.

During the 30 minutes prior to the feeding the gall bladder pressure fluctuated as shown in the chart. Shortly after the first ingestion of food the column of bile rose to 200 mm. and gradually sank again to 120 mm. 5 minutes later it began to rise, again attaining the 200 mm. level and again sinking, this time to 130 mm. 25 minutes after the taking of food it showed another increase, this time to 220 mm., which pressure was maintained for 5 minutes. The tube

was now opened for a few seconds during which 3 cc. of bile flowed from it. The pressure fell to 100 mm. but soon rose again to 220 mm. and 5 minutes later to 260 mm. The dog was very quiet during the entire period of the observations.

The 3 cc. of bile collected was dark and viscid and contained 1.47 mg. of bilirubin per cc., this despite the fact that there was an admixture with liver bile derived from the left central lobe of the liver; the 7.3 cc. of liver bile from the remainder of the liver obtained during the 35 minute interval between the taking of food and the collection of bile from the gall bladder was light and limpid and contained but 0.55 mg. of the pigment per cc.

The cross-hatched columns in the text-figure show that there was a slightly increased output of bile by the liver after the feeding.

It is noteworthy that in these two experiments, and in all similar ones as well, there was no sudden gush of bile from the liver, the increase in bile secretion after feeding being very gradual. The abrupt rises in the column of bile in the manometer connected with the gall bladder can therefore have been due only to pressure changes within the viscus. Are they the result of true gall bladder contractions or of extraneous pressure factors?

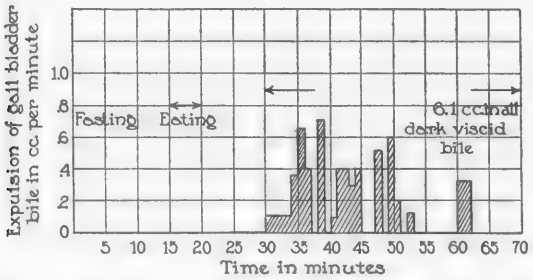
The observed phenomena cannot be the effect of a generalized increase in abdominal pressure consequent upon an increase in the gastric contents; for it is well known[26] that this pressure remains constant during the taking of food unless enormous quantities are eaten. Kelling[27] has shown that in the case of the dog more than 300 gm. of food can be taken without increasing the intraabdominal pressure, owing to a compensating relaxation of the muscles of the abdominal wall. In some of our experiments (as that shown in Text-fig. 5) the column of bile in the tube connected with the gall bladder rose abruptly when the animal had taken not more than 150 gm. of food, into a stomach previously empty. In several experiments, like that charted in Text-fig. 6, the rhythmic recurrence of abrupt increases in pressure within the gall bladder, in the absence of any further ingestion of food, rules out the possibility that a generalized increase in intraabdominal pressure was the direct cause of the phenomenon. Furthermore in several other instances, none of which have been charted, increases in pressure within the gall bladder did not occur until 10 to 15 minutes after food had been

[26] Cannon, W. B., The mechanical factors of digestion, London, 1911.
[27] Kelling, G., *Z. Biol.*, 1903, xliv, 161.

eaten. In an experiment to be described below (Text-fig. 7), the tube leading to the gall bladder was allowed to drain freely into a sterile graduate. Even under these conditions no bile drained from the organ for 10 minutes after food had been eaten. Obviously in this instance and in those cited above increases in the gastric contents had caused no increase in pressure within the gall bladder.

The pressure changes within the viscus endured so long, for example nearly 10 minutes in the case shown in Text-fig. 5, 5 or 10 minutes in that of Text-fig. 6, and occurred at such lengthy intervals, that they cannot be ascribed to the transmission of rhythmic peristaltic pressure changes from the other viscera.

Obviously then, these findings can have come about through



TEXT-FIG. 7. Expulsion of bile from the gall bladder during gastric digestion. The intermittency of the flow is evidence of separate increases in pressure within the organ such as have been charted in Text-fig. 6.

nothing but prolonged forceful contractions of the gall bladder, following the stimulus of taking food, and occurring repeatedly during early digestion. They had the character of rhythmic smooth muscle contractions.

In an accessory experiment in which the same animal was employed, as in the experiment described in the previous protocol, the amount of bile emerging from the intubated gall bladder was studied, not the pressure exerted upon it.

Dog 16, Text-fig. 7. 16 days after a "triple intubation" and following a 24 hour fast the tube leading to the gall bladder was opened, so that it might drain freely into a sterile graduate. For a period of half an hour no bile appeared. 200 gm. of food was then offered to the animal and eaten in 5 minutes. The

usual expulsion of bile at the first taking of food did not occur; and only 15 minutes later did bile flow from the open end of the tube. The amount collected during each minute thereafter was recorded, and is shown in the text-figure. It appeared in sudden gushes, each enduring but a few seconds and followed by a slow ooze from the open end of the tube. These ejections persisted intermittently for 35 minutes and then ceased. As the chart shows, no bile was obtained during certain periods. Of these there were four of 1 minute each, one of 2 minutes, and one of 7 minutes. 6.1 cc. of bile in all were expelled in this way.

At autopsy, performed a week later, after the animal had been killed with chloroform while in excellent health, the bile ducts were all found normal in appearance, and no obstruction was noted. There was a small duct secreting bile into the hepatic duct above the point of intubation. It was found to drain about 40 gm. of liver and, from what is known of the rate of bile secretion in the dog, can have provided but very little of the 6.1 cc. of bile. This latter had the general character of "gall bladder" bile, being dark and viscid and with a pigment content of 1.94 mg. of bilirubin per cc.

The intermittent voidings of bile recorded in the chart suggest that individual contractions of the gall bladder took place at intervals throughout a period of half an hour during gastric digestion. As just mentioned a duct from a small proportion of the liver secreted its bile into the hepatic duct above the point of intubation. But the voidings can hardly be ascribed to the influence of the food stimulus to increase the formation of liver bile, a part of which was voided through the tiny duct. For, as shown in Text-fig. 6, the increase in bile formation which occurred after the taking of food by this same animal was slow and gradual, as in all our feeding experiments. Moreover the bile was voided intermittently from the tube leading to the gall bladder, not continuously, and it had the general characteristics of "gall bladder" bile, being dark and viscid.

Reciprocal Activities of the Gall Bladder and the Musculature about the Lower Portion of the Common Duct.

The assumption that the gall bladder contracts and the sphincter of the common duct relaxes at the same time has been much disputed in the past.

Doyon[28] was the first to believe that he had shown such an occurrence. He experimented with the anesthetized dog. Rost[29] more recently, through ob-

[28] Doyon, M., *Arch. physiol. norm. et path.*, 1894, vi, series 5, 19.
[29] Rost, F., *Mitt. Grenzgeb. Med. u. Chir.*, 1913, xxvi, 711.

servations on the voidings of bile in unanesthetized dogs with the Pavlov biliary fistula,[30] came to the same belief. Meltzer[31] accepted such an occurrence as constituting a special instance of the law of reciprocal innervation.

We have shown in an accompanying paper that at the first perception of the nearness of food and also upon its ingestion the physiological resistance to the passage of bile into the gut relaxes. In the present contribution we have presented evidence that the same stimulus causes a discharge of bile from the gall bladder, presumably by its contraction. In experiments now to be reported we have attempted to determine whether there is a relationship in time between the two phenomena. There is one. They occur synchronously. A type experiment follows.

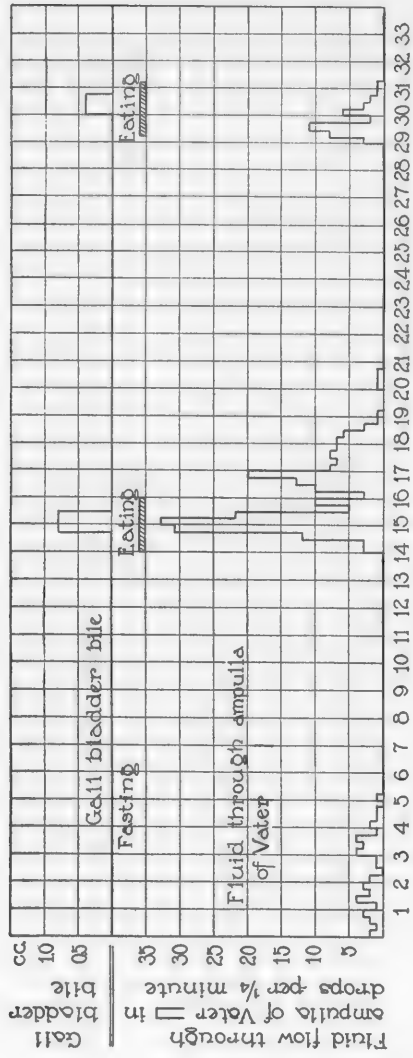
Dog 19, male, weight 12 kilos, Text-fig. 8. 2 weeks prior to the experiment a "triple intubation," under ether anesthesia, with insertion of the third cannula directly in the cystic duct, was followed by prompt and complete recovery. The dog remained healthy and active, with the bile draining as normally save for the detour.

After a fast of 30 hours the tube connecting with the lower common duct was joined to the "flow" manometer, described in the accompanying paper,[1] for measuring the flow of fluid through the lower common duct at a constant pressure. The bile columns in this manometer were adjusted to exert a pressure equal to that of a 170 mm. column of bile. The flow of fluid was estimated by counting the drops emerging from the nozzle in periods of 15 seconds. The tube connected with the gall bladder was allowed to drain into a sterile, vertical tube of such length that no bile could be collected from it into a sterile graduate at its end until the pressure of a 200 mm. column of bile had been overcome. For the sake of simplicity the amount of liver bile drained by the third tube and collected during the period of the experiment is not recorded in the chart.

During an initial 15 minute period of observation no bile was forced out of the gall bladder and but little passed from the flow manometer into the duodenum.

Food was then given for 2 minutes. Almost at once, 5 to 10 seconds after the first swallow, the bile column began to rise in the vertical tube connected with the gall bladder. In 45 seconds, as shown in the chart, dark viscid "gall bladder" bile was forced into the collecting graduate at the end of this vertical tube, against the 200 mm. of pressure, and it continued to flow for 45 seconds more, delivering 0.6 cc. in all. Then the bile column in the tube fell to the 180 mm. level, so that bile no longer entered the graduate at its end, a finding not shown in the chart. During the next 2 minutes the column fell slowly to the

[30] Pavlov, S. P., *Ergebn. Physiol.*, 1902, i, 1. Abt., 246.
[31] Meltzer, S. S., *Am. J. Med. Sc.*, 1917, clxiii, 469.



TEXT-FIG. 8. Upon feeding there occurs simultaneously an expulsion of bile by the gall bladder and a lessening in the resistance to the passage of bile to the duodenum.

The upper curve shows when bile is expelled from the gall bladder against the resistance offered by a column of bile 200 mm. high, and also the amounts expelled. The lower curve records the periods of decrease in the resistance to the passage of bile into the intestine as evidenced by a flow of fluid to the gut.

original level of about 100 mm. Simultaneously with the intake of food, as shown in the chart, the flow to the duodenum occurred indicating a decrease in the resistance thereto and the flow kept up for some time thereafter, its rate indicating a progressive lessening in the resistance, one most pronounced at the precise period when bile was being ejected from the gall bladder. The flow ceased in 5 minutes but 10 minutes later recurred again upon a second brief feeding, as did also the expulsion of bile from the gall bladder. This time the greatest rapidity of flow was not quite synchronous with the expulsion from the gall bladder.

At autopsy 9 days later the bile ducts were found normal in appearance, the cannulas and tubes were open and intact.

In this experiment the pressure within the gall bladder increased sufficiently to force bile out of the organ against the weight of a column of bile 200 mm. in height. No similar expulsion occurred until food was again offered. These pressure increases were synchronous with a decrease in the resistance to the passage of bile to the gut. That only small amounts of bile were obtained from the gall bladder can be accounted for by the fact that it was necessary for the organ to do more work forcing bile out against this artificially high resistance than would be needed to expel the fluid through the normal channels under the condition of lessened resistance prevailing at the time.

In our five dogs with "triple intubation" this experiment was repeatedly carried out, with in each case the same result. Always upon taking food, frequently at the mere perception of it,[1] there was a sudden synchronous decrease in the resistance to the passage of bile into the intestine and an increase in the pressure within the gall bladder as shown by the expulsion of bile. The pressure increase on the part of the gall bladder did not at once reach its maximum, a point shown in Text-figs. 5 and 6, but endured at times several minutes and served to expel several cc. of bile from the viscus, when the resistance thereto was slight (Text-fig. 7).

DISCUSSION.

The observations recorded in this and the accompanying paper would appear to possess a special worth because they were made under controlled circumstances in the healthy, unanesthetized dog.

We have been able to prove that upon the taking of food some portion of the gall bladder contents is expelled forcibly by contraction of the viscus. But more than this, we have demonstrated the existence of the long supposed and equally as long disputed reciprocal relationship between gall bladder activity and that of the musculature about the lower end of the common duct, as expressed in resistance to bile flow. At the same time that the gall bladder contracts the resistance to the flow of bile into the gut markedly lessens, thus making for a more effective discharge of the gall bladder contents.

The discharge of bile into the duodenum may be thought of as chiefly dependent upon the interaction of three factors, the tonus of the muscles about the lower common duct, the activity of the gall bladder, and the pressure of bile secretion. To make clear the interaction of these in effecting the flow of bile to the intestine let us consider their activities in connection with the taking and digestion of food. The demonstration of the slight differences in pressure causing bile to flow, first toward the intestine, then toward the gall bladder, reveals a truly exquisite mechanism.

In a paper published with this one we have described certain physiological variations in the resistance offered to the passage of bile into the intestine. The "normal" resistance, 4 to 12 hours after a feeding, is sufficient to hold back a column of bile 100 to 120 mm. in height. The resistance increased, during fasting periods often supporting a column of bile 200 to 250 mm. high. Promptly at the mere perception of food and again upon its ingestion a decrease in this resistance was noted, bile flowing through the ampulla of Vater at 50 mm. pressure or even less. The reaction was so immediate that one must suppose it to have been reflex in nature. This decrease in resistance was but transient for the latter soon increased after food had entered the stomach and often became high enough during a brief period to support a 250 mm. column of bile. After a period varying from 10 to 30 minutes the resistance decreased and it fluctuated from high to low as gastric digestion proceeded.

The two other factors which affect the escape of bile into the duodenum—the pressure of bile secretion and the gall bladder activity —have been described in this present paper. We have shown in fasting animals with gall bladder cut off from the duct system that

bile is secreted by the liver at a constant rate into a manometer tube until the column of bile has risen in the face of the progressive obstruction to 300 mm., when secretion abruptly ceases. If, however, the gall bladder is still connected with the ducts the pressure of bile developing within the biliary tract as measured by a manometer connected with the common duct remains for some hours below that of a column of bile 175 mm. in height and is usually equivalent to that of a bile column of 100 to 150 mm. When food is eaten there soon occurs within the gall bladder an increase in pressure such as would result from a contraction of smooth muscle, and with it an actual partial emptying of the organ, an activity synchronous with a relaxation of the resistance to the passage of bile to the gut. The phenomenon recurs at intervals during the process of gastric digestion.

It is of importance to note that the pressure developed within the gall bladder in these experiments was amply sufficient to force bile through the ampulla of Vater against the resistance of the muscles in this region, save when the latter were in that temporary state of high tonus, enduring 10 to 30 minutes, which closely follows their immediate relaxation upon the taking of food. It is probable that during this brief period but little bile entered the intestine. Even under these conditions, however, the pressure exerted by the gall bladder contractions about equalled the resistance offered. Later much bile may have entered the intestine, for these contractions, as we have shown, occurred repeatedly during gastric digestion.

From these observations it is possible to correlate the biliary factors which are responsible for the discharge of bile into the duodenum with the taking and digestion of food.

During fasting periods bile is prevented from entering the intestine by the high resistance of the muscles about the lower common duct. The force of secretion is thus directed toward the filling of the gall bladder, which, by its concentrating activity, is able to admit and store large amounts of bile. As result the liver may secrete bile continuously without its appearance at the ampulla of Vater, and the development of a high pressure within the ducts is prevented.

The escape of bile into the intestine occurs promptly at the first ingestion of food, practically ceases then for a short period, and later

continues at intervals during the process of gastric digestion. Whether the actual ejection of bile is synchronous with and perhaps dependent upon the gushes of acid chyme through the pylorus cannot be said from our observations. That some relationship does exist is suggested by the fact that the resistance to bile passage into the intestine fluctuates during gastric digestion as further by the intermittent character of the expulsion of bile from the gall bladder.

The contractions of the gall bladder we studied were slow and endured often for several minutes, sometimes coming on quickly, sometimes slowly and wearing off gradually. They were such as to slightly more than double the pressure within the organ. It is of importance to note too that only a little bile was ejected at any one time. This fact may readily account for the failure of previous workers to observe contractions of the gall bladder when they have been sought by methods rendering the organ visible. Of course in such procedures the effects of the operations and the anesthetics employed have militated against success. To explain a supposititious discharge of gall bladder contents in the absence of visible contractions of the organ many authors have invoked the inspiratory increase in pressure on the viscus. Recently, on the basis of new work, Winkelstein[10] and Winkelstein and Aschner[32,33] have concluded that respiration is the "motor of the gall bladder." During our observations we sometimes noted considerable fluctuations in the pressure within the biliary tract traceable obviously to the respiratory movements. While the dog was breathing or panting quietly the pressure changes were not more than a few mm., scarcely enough to affect the entrance of bile into the gall bladder or its exit therefrom. Usually, the column of bile in the manometer connected with the gall bladder rose and fell about 10 to 15 mm. with each breath. Occasionally, however, on deep inspirations, the pressure often increased 20 to 30 mm. The retching movements preparatory to vomiting raised it to 600 mm., and the straining incident to defecation was once seen to cause a rise in the column of 50 mm. It is to be remembered however that these pressure alterations affected not only the gall gladder but the gut which might

[32] Winkelstein, A., and Aschner, P. W., *Am. J. Med. Sc.*, 1924, clxviii, 812.
[33] Winkelstein, A., and Aschner, P. W., *Am. J. Med. Sc.*, 1926, clxxi, 104.

receive bile from it and that under these circumstances no flow could occur. As Doyon has pointed out,[28] it does not seem that respiratory changes in pressure can be held responsible for the normal discharge of the gall bladder contents. The average respiratory increase in pressure would seem to be insufficient to cause a flow of bile through the ampulla of Vater unless the musculature about the lower common duct is much relaxed as it is just after eating. At such times the mere pressure of bile within the ducts is sufficient for the task, as we have shown in the preceding paper.[1]

The facts reported here have an important clinical significance. That the normal resistance to the passage of bile through the ampulla is connected in some way with gall bladder function is sufficiently shown by the absence of this resistance in species lacking the viscus[34] and also by the dilatation of the ducts[29] and the breaking down of the resistance which occurs in individuals from which the organ has been removed surgically.[7] Cholecystectomy destroys the mechanism governing the intermittent expulsion of bile that we have described. The fact is of significance for it bears upon the origin of the digestive disturbances occurring in patients after removal of the gall bladder.

A further point deserves mention. The stimulus of food brings about a physiological bile drainage, by relaxation of the muscles about the lower common duct with contraction of the gall bladder. The value of frequent administrations of food to patients when it is desired to promote such drainage is evident. It is conceivable that such feedings will be more efficacious if the food is sufficiently acid to bring about the extreme relaxation of the musculature about the lower common duct noted under experimental conditions when acid is fed.[1,35]

SUMMARY.

After feeding a dog, forceful contractions of the gall bladder occur that are sufficient in strength to expel part of the contents of the viscus against a considerable pressure resistance.

The pressure within the gall bladder of a healthy, unanesthetized

[34] Mann, F. C., *J. Lab. and Clin. Med.*, 1919–20, v, 107.
[35] Cole, W. H., *Am. J. Physiol.*, 1925, lxxii, 39.

dog fasted 24 to 48 hours is usually about equal to a column of bile 100 mm. high. After a few swallows of food there is a rapid increase in the pressure to more than 200 mm. with a gradual fall in it again, and repeated similar rises and falls occur thereafter. The gall bladder contractions responsible for these alterations are accompanied by a lessening in the resistance to the passage of bile to the intestine, a resistance which is maintained by the muscles at the lower end of the common duct. There would appear to be a reciprocal response on the part of the two structures to the one stimulus.

The maximum pressure developing within the temporarily obstructed biliary tract in an animal with the gall bladder excluded about equals that of a column of bile slightly more than 300 mm. in height. The taking of food acts as a stimulus on the rate of bile secretion, but does not alter the maximum secretion pressure. When the gall bladder is connected with the duct system, obstruction does not lead until after some hours to the development of a pressure of more than 100 to 150 mm. within the biliary tract,—that is to say the pressure does not rise above the normal. Its failure to rise further is referable to the activity of the gall bladder to store and concentrate the bile as secreted.

The physiological and clinical significance of these findings is discussed.

STUDIES ON THE OXIDATION AND REDUCTION OF IMMUNOLOGICAL SUBSTANCES.

I. Pneumococcus Hemotoxin.*

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INTRODUCTION.

Certain general principles which were established in previous papers (1, 2) on the biological oxidation-reduction of hemoglobin are reviewed below since they are to be utilized in the present series of studies.

Methemoglobin may be considered as the "inactive" form of hemoglobin, in that it no longer combines with oxygen or carbon monoxide. The essential difference between hemoglobin, the "active" blood pigment, and methemoglobin, its "inactive" oxidation product, is the change of the ferrous iron of the molecule to the ferric state. The conversion of hemoglobin to methemoglobin thus, is a "true" oxidation in the electronic sense (3) and must be distinguished from the process of "oxygenation" involved in the formation of oxyhemoglobin.

The oxidation to methemoglobin may be brought about by biological oxidizing agents, and is a reversible process. Both the oxidation ("inactivation") and the reduction ("reactivation") seem to be induced by the same system; the substances or biological systems which in the presence of molecular oxygen, bring about the oxidation of the hemoglobin, induce the reverse process if air is excluded. Apparently these substances, if not disturbed by the presence of oxygen, are essentially reducing agents, but when oxygen is present, they are changed to oxidizing agents (peroxides or "activated oxygen") which bring about the oxidation of other more difficultly oxidized substances such as hemoglobin. All of the hemoglobin oxidations of this type proceed most rapidly at an oxygen tension of approximately 20 mm., which permits the formation of the oxidizing agents but which also permits

* The major portion of the investigations reported in this and three following papers was carried out in the Department of Bacteriology and Immunology of Harvard University Medical School during the author's tenure of a Traveling Fellowship granted by the General Education Board. I wish to express my appreciation of the courtesies and laboratory facilities extended to me by Dr. Zinsser and his staff throughout the year spent in his department.

over half of the total hemoglobin to exist in the reduced (deoxygenated) state. The "spontaneous deterioration" of hemoglobin to methemoglobin is also an oxidation process and it is influenced by the tension of molecular oxygen in exactly the same way. This gradual deterioration of Hb (so called "spontaneous MetHb formation") which always occurs when blood or Hb solutions are stored under the usual conditions outside the body, can be inhibited or wholly prevented if the hemoglobin is stored in a sealed system in the presence of a biological reducing agent. Thus, when air is excluded, the stability of the active blood pigment is actually increased by the presence of the same biological substances which rapidly oxidize it in the presence of air.

A study has now been undertaken in which it is attempted to apply these principles, as outlined for hemoglobin, to the oxidation and reduction of substances of importance in immunology. It seemed that such a study might well be begun with experiments on bacterial hemotoxins. These substances, which apparently are true antigens, although themselves of relatively little importance, have proved valuable objects of study in the historical establishment of many of the general principles of immunology. The hemotoxin of Pneumococcus was the particular one chosen as the subject of the first investigation because the mechanism of the oxidation of this bacterial substance had been established in an earlier paper (3).

The term bacterial *hemotoxin* has been proposed by Přibram (5) to distinguish the bacterial lysins which exhibit antigenic properties from other substances of bacterial origin which although possessing hemolytic properties are not antigenic. It is also important to avoid confusing the hemotoxins with the so called immune hemolysins. The hemotoxins are substances which are specifically (or primarily) toxic for red blood cells, just as diphtheria toxin and tetanus toxin are, respectively, selective for other tissue cells. Again, like the "true" toxins, the hemotoxins are *antigenic*, or more properly speaking "antitoxinogenic," in that when injected into animals they induce the formation of a neutralizing antihemotoxin (analogous to antitoxin). The hemolysins, on the other hand, are "sensitizing" *antibodies* induced by the injection of a primarily non-toxic antigen (red blood cells), and, unlike the hemotoxins, their species-specific hemolytic action is dependent upon the cooperative mechanism of the sensitizing hemolysin and alexin (complement).

Pneumococcus hemotoxin has been described by a number of workers (6, 7) but its first complete study was made by Cole (8) in 1914. It is a true antigen and when injected into animals causes the formation of an antihemotoxin, an antibody which neutralizes the hemotoxin (8). Unlike most bacterial hemotoxins, the hemotoxin of Pneumococcus is an endocellular substance liberated into the culture fluid only upon disintegration of the cells. As might be expected, the

detection of active hemotoxin in filtrates of pneumococcus cultures depends not only upon the liberation of the lysin by cell autolysis, but also upon its protection from air (9). There seems to be no relation between the virulence of the strain of Pneumococcus and its hemotoxin-producing capacity. By comparisons of the strains in the collection at the Hospital of The Rockefeller Institute, we have found that the most virulent strains frequently are weak lysin producers, and that avirulent strains as often as not produce larger amounts of the hemotoxin.

It is evident from the foregoing that in pneumococcus hemotoxin we are dealing with a specific antigenic constituent of the bacterial cell.

EXPERIMENTAL.

Methods.—Sterile pneumococcus extracts (10) supplied the hemotoxin. The term "reduced" extract, as used in this paper, refers to pneumococcus extracts which have been protected from oxidation. The term "oxidized" extract denotes extracts in which the hemotoxin has been oxidized by exposure to air in unagitated Erlenmeyer flasks. The pneumococcus extracts employed throughout the present study consisted of the filtered extract of a concentrated suspension of pneumococcus cells which had been disrupted by repeated freezings and thawings. This type of extract (the "complete system" type) (10–13) contains easily oxidized substances which upon exposure to air form oxidizing agents which inactivate the hemotoxin. The differences between this type of pneumococcus extract and the so called "incomplete system" type of extract have already been described (11). The hemolysis tests were made in salt suspensions of well washed sheep or horse erythrocytes. The extracts which were used in most of the experiments were prepared by Dr. Louis A. Julianelle, of the Hospital of The Rockefeller Institute for Medical Research.

"Reactivation" of Oxidized Hemotoxin by the Reducing Activity of Bacteria.

In a preceding paper (14) it was shown that the methemoglobin formed by the oxidizing action of pneumococci or of sterile pneumococcus extracts, could, in the absence of air, be reconverted to hemoglobin by the reducing action of the bacteria. Hence, experiments were made to determine if the same biological reducing agents can reconvert the hemolytically inactive oxidation product of pneumococcus hemotoxin to the original active form. Accordingly, bacteria were added to pneumococcus extracts in which the hemotoxin had previously been inactivated by oxidation. The mixtures were sealed

and incubated for several hours; after the period allowed for reduction, the bacterial cells were removed by centrifugation, and the bacteria-free supernatant of the mixture was titrated for its hemotoxin content. Controls were included to prove that the bacteria used as reducing agents were devoid of hemolytic activity.

Pneumococcus hemotoxin inactivated by oxidation was obtained by exposing an active pneumococcus extract (Type III) to air for 3 hours at 38°C.

Cultures of *B. coli* and of Anaerobic Bacillus *T* (an anaerobic organism isolated from a wound and morphologically resembling *B. tetani*) were grown in 50 cc. centrifuge tubes under vaseline seal; these cultures were centrifuged and the bacterial cells were suspended in 1.0 cc. of the supernatant broth.

TABLE I.

"Reactivation" of Oxidized Pneumococcus Hemotoxin by the Anaerobic Action of Bacteria.

Amount of pneumococcus extract.	Original (reduced) lysin.	Oxidized lysin.	Oxidized lysin after reduction by *B. coli*.	Oxidized lysin after reduction by anaerobic bacilli.	Controls on bacterial reducing agents.
cc.					
0.10	++++	−	++++	++++	−
0.05	++++	−	++++	++++	−
0.04	++++	−	++++	++++	−
0.02	++++	−	++++	++++	−
0.01	++++	−	++++	++++	−

The following test mixtures were prepared:

(1) 0.5 cc. oxidized pneumococcus extract + 0.5 cc. broth.
(2) 0.5 " " " " + 0.5 " suspension of *B. coli*.
(3) 0.5 " " " " + 0.5 " " " Anaerobic Bacillus T.
(4) 0.5 " broth + 0.5 cc. suspension of *B. coli*.
(5) 0.5 " " + 0.5 " " " Anaerobic Bacillus T.

These mixtures were sealed with vaseline and incubated at 38°C. for 1½ hours, after which time they were centrifuged at high speed. The hemolytic activity of the supernatants of these mixtures was then titrated. Mixtures (4) and (5), comprising controls on the bacterial reducing agents employed, were always negative whether or not the bacterial cells were removed from the test samples added to the erythrocyte suspensions. The results of the titrations of these mixtures ((4) and (5)) are presented jointly in the protocol under the heading "Controls on bacterial reducing agents."

The protocol of a typical experiment is presented in Table I.

The results (Table I) of experiments such as that described indicate that the inactive, oxidation product of pneumococcus hemotoxin may be reconverted to an actively hemolytic substance by the action of certain bacteria which are not themselves hemolytic. A sufficient number of control tests have been made to prove that the hemolytic substance produced by the action of these bacterial agents upon oxidized pneumococcus extract is not yielded by the action of the same agents upon the broth medium.

Identity of the Active Hemotoxin in the Original Reduced Extracts and in the Oxidized Extracts "Reactivated" by Reduction.

Experiments were designed to prove that the hemolytic substance yielded by the action of bacterial reducing agents upon oxidized pneumococcus extracts was identical with the original hemotoxin present in the "reduced" pneumococcus extract. These experiments included comparisons of the heat lability of the lysins and tests of their immunological neutralization.

A. Comparison of Heat Lability.—Although bacterial hemotoxins, as a class, are relatively heat-labile, certain degrees of difference in lability exist between the lysins of different bacterial origin, as will be shown in subsequent papers. The hemotoxin of Pneumococcus stands out as an exceptionally heat-labile substance. When heated in narrow, thin walled tubes, it is entirely inactivated by 90 seconds exposure to 55°C. This is a considerably greater heat lability than that of most other hemolytic substances. It is conceivable that if the lysin yielded in the preceding experiment differed from the one present in the original reduced extract, it might be found to possess a higher degree of heat resistance than the original pneumococcus hemotoxin.

Comparative heating tests were made on 0.6 cc. of reduced pneumococcus extract and 0.6 cc. of the supernatant of the reduced mixtures of oxidized extract and bacterial cells (mixtures analogous to (2) and (3) in the preceding experiments). The test liquids were placed in narrow thin walled tubes, sealed with vaseline, and heated at 55°C. for 90 seconds in an agitated water bath.

B. Specific Immunological Neutralization.—Tests of the neutralization of the hemolytic substance by the antihemotoxin in pneumococcus immune serum were made as follows: 3 hemolytic units of the original hemotoxin, and the same amount of the hemolytic substance produced by the action of the bacteria upon the oxidized extract, were diluted to 1.5 cc. with salt solution; 0.005 cc. of normal horse serum and 0.005 cc. of the serum of a horse immunized against Type I Pneumococcus were added to separate tubes of each of the lysins; after 45 minutes incubation of the mixtures, 0.5 cc. of 20 per cent sheep cells was added to each tube. (The

extracts used as source of hemotoxin were of Type III pneumococci. Since the antihemotoxin is not "type-specific" a heterologous immune serum was used to avoid the specific precipitation of the Type III "S" substance also present in the extracts.)

The results of both of these experiments are given in Table II.

As shown in Table II, the hemolytic substance produced by the anaerobic action of the bacteria upon oxidized pneumococcus hemotoxin possesses heat lability comparable to that of the original reduced hemotoxin. This fact merely suggests that the two are identical. More convincing proof is furnished by the immunological neutralization of the "reactivated" hemotoxin by the antihemotoxin present in pneumo-

TABLE II.

Identity of the Active Hemotoxin in the Original Reduced Extracts and in the Oxidized Extracts "Reactivated" by the Anaerobic Action of Bacteria.

	Heat lability.		Neutralization by anti-pneumococcus serum of heterologous type.	
			Hemolysis by 3 hemolytic units previously incubated with 0.005 cc. serum.	
	Hemolysis by 0.01 cc. unheated extract.	Hemolysis by 0.1 cc. extract heated 90 sec. at 55°C.	Normal serum.	Immune serum.
Original active hemotoxin in reduced extract	++++	−	++++	−
Hemotoxin inactivated by oxidation and "reactivated" by reduction	++++	−	++++	−

coccus immune serum. This antibody is specific and does not neutralize the lytic substances produced by other bacteria.[1]

Failure to "Reactivate" Hemotoxin Which Has Been Inactivated by Heat.

Experiments were next made to determine if the inactive product obtained by heating pneumococcus hemotoxin can be "reactivated" by the reducing action of bacterial cells. Obviously, the lysin should be heated as little as possible, else it may be so changed chemically that it cannot be reactivated. It seemed probable from the studies made of heated hemoglobin (1) that "reduced" pneumococcus hemo-

[1] Unpublished experiments to be presented in a subsequent paper.

toxin heated anaerobically may yield inactive products which cannot be "reactivated" by the reducing agents which serve to "reactivate" the oxidation product of the hemotoxin.

"Reduced" (active) hemotoxin was heated anaerobically for a period just sufficient to destroy its activity. The heating was carried out exactly as described in the preceding experiment. Another portion of the same extract was inactivated by exposure to air. The reduction tests were made by use of mixtures analogous to those described in the experiments reported in Table I. The results are presented in Table III.

From Table III, it is obvious that the biological reducing agents which serve to "reactivate" the oxidation product of pneumococcus

TABLE III.

Attempt to "Reactivate" Heat-Inactivated Hemotoxin.

Amount extract.	Hemotoxin in reduced pneumococcus extract.		Hemotoxin in unheated oxidized extract (before action of bacteria).	Hemotoxin in unheated oxidized extract (after action of bacteria).	Hemotoxin in heated oxidized extract (after action of bacteria).
	Unheated.	Heated.			
cc.					
0.06	++++	−	−	++++	−
0.04	++++	−	−	++++	−
0.03	++++	−	−	++++	−
0.02	++++	−	−	++++	−
0.01	++	−	−	+	−

hemotoxin, fail entirely to "reactivate" the inactive products formed in the anaerobic heating of the reduced hemotoxin. These results serve as a valuable check on the preceding experiments, and indicate, moreover, that the "inactive" products formed in the oxidation of the hemotoxin are quite different from those formed when the hemotoxin is destroyed by heat. Since different products are formed, the reactions involved in the inactivation of the hemotoxin by oxidation and by heat must be of different nature, the inactivation by oxidation apparently being similar in nature to the reversible change of hemoglobin to methemoglobin, while the inactivation by heat is comparable to the destruction of hemoglobin to irreversible blood pigment derivatives.

"Reactivation" of Oxidized Pnemococcus Hemotoxin by Treatment with Sodium Hydrosulfite.

It seemed probable from the preceding experiments, that the "reactivation" of the oxidized hemotoxin by the anaerobic action of bacterial cells represented a reduction process. More definite evidence was obtained by the following tests which are similar to those previously described, with the exception that a chemical reducing agent, sodium hydrosulfite, was substituted for the biological reducing agents previously employed.

Weighed amounts of sodium hydrosulfite ($Na_2S_2O_4$) were placed in large test-tubes. Solutions of the required strength were made in measured amounts of 0.1 M, pH 7.5 phosphate buffer mixtures. The solutions were made up fresh for each test and were used within less than 5 minutes after their preparation.

Preliminary tests were made of the possible hemolytic action of the hydrosulfite itself. If solutions of hydrosulfite were added to broth in sufficient strength to give a concentration of 5 per cent in the reduction mixture, the addition of from 0.02 to 0.10 cc. of the mixtures to 1 cc. of blood cell suspension was not without effect. Upon incubation of the blood cell suspensions containing this amount of hydrosulfite, a part of the blood pigment was converted to methemoglobin. This is in conformity with the reports of Conant and Fieser (15) on the action of the oxidation products of the hydrosulfite. As in the case of the biological reducing agents described in previous papers (1, 2), the hydrosulfite, which essentially is a reducing agent, forms oxidation products in the presence of air which oxidize the blood pigment. This phenomenon is important in tests such as are to be described, from two points of view. First, the "methemoglobinized" red blood cell may be much more difficultly hemolyzed than the unaltered cell. (Reports to this effect in the literature have been partially confirmed by incomplete experiments of our own.) Second, there is also a possibility that the same oxidizing agents which oxidize the hemoglobin in the presence of air, may also oxidize the hemotoxin if the reduction mixture is freely exposed to air *before* its addition to the blood cells.

However, both of these difficulties are readily overcome. Amounts of hydrosulfite too small to injure the blood cell suffice to reduce the oxidized hemotoxin.

The possible injurious action of the aerobic oxidation products of hydrosulfite upon the hemotoxin can be avoided by adding the samples of the reduced mixtures to the red blood cells with a minimum exposure of the reduced mixture to air. Probably the small samples of the mixtures taken for the hemolysis tests include insufficient hydrosulfite to injure the hemotoxin, or possibly the hemotoxin after combination with the red blood cell is difficult to destroy (in analogy with the relative stability exhibited toward oxidizing agents by hemoglobin after combination with oxygen or carbon monoxide) (2). If sufficiently large amounts of the reduction mixtures are added to the blood cells, enough hydrosulfite is included to reduce the oxyhemoglobin. This, however, does not seem to interfere significantly with the hemolytic titres.

In the experiments tabulated in the protocol to be presented in Table IV the following detailed procedure was used.

0.2 cc. of pneumococcus extract previously inactivated by oxidation, diluted to 1.0 cc. with 0.1 M phosphate solution (pH 7.5), was placed in a series of tubes.

TABLE IV.

"Reactivation" of Unheated Oxidized Pneumococcus Hemotoxin by Reduction with Sodium Hydrosulfite.

	Hemotoxin titrations.		
	0.03 cc.	0.02 cc.	0.01 cc.
Oxidized extract untreated	−	−	−
Oxidized extract treated with $Na_2S_2O_4$	++++	++++	++
Heated extract treated with $Na_2S_2O_4$	−	−	−

Another series of tubes containing 0.2 cc. of extract inactivated by heat and similarly diluted, and a third series containing broth instead of pneumococcus extract were prepared.

To these tubes was added 0.2 cc. of various dilutions of sodium hydrosulfite dissolved in 0.1 M phosphate solution (pH 7.5). These mixtures were sealed with vaseline. After allowing time for reduction, varying from 5 minutes in the case of the higher concentrations of the reducing agent to 30 minutes in the case of the lower concentrations, the hemolytic activity of the reduced mixtures was titrated. The samples were added to the red blood cells immediately after the removal of the seal. The reduction, of course, is dependent upon the pH, the temperature, and the time, as well as the concentration of hydrosulfite. The pH was maintained at 7.5, the temperature was approximately 25°C., and the time allowed for reduction was never over 30 minutes. The concentrations of $Na_2S_2O_4$ in the reduction mixtures reported in the table ranged from 1.0 to 0.008 per cent.

The hemolytic test mixtures were incubated for 1 hour at 38°C. The test mixtures were then centrifuged and the hemolytic titres recorded.

The results are given in Table IV.

The results (Table IV) show that the inactive product formed by the oxidation of pneumococcus hemotoxin may be "reactivated" by chemical reducing agents as well as by the reducing action of bacteria. Reducing agents convert the inactive products formed during exposure of the hemotoxin to air, to the original active hemotoxin, but they do not "reactivate" the products formed by the anaerobic heating of the active hemotoxin.

Attempts to "Reactivate" Oxidized Pneumococcus Hemotoxin by the Reducing Action of Sterile Animal Tissues.

In previous studies (12) it has been shown that methemoglobin is formed by action upon hemoglobin by the same agents responsible for the oxidation of the hemotoxin. The oxidized, "inactive" blood pigment can subsequently be converted to "active" hemoglobin by the reducing action of sterile animal tissues *in vitro* (14). It seemed probable that the reducing action of animal tissues would likewise "reactivate" the oxidation product of the hemotoxin. The importance of such an action of the tissues is an obvious one, since the hemotoxin is an antigen of bacterial origin.

A limited number of tests have been made by adding sterile, rabbit testicle to oxidized pneumococcus hemotoxin. In no case was an "active" lysin obtained. The negative results of these experiments, however, do not rule out a reducing action of animal tissue to "reactivate" the oxidized hemotoxin. It is quite possible that the hemotoxin was actually reduced but was combined with the lipoids of the tissues. The literature furnishes many reports of the "inactivation" or neutralization of bacterial hemotoxins by tissues *in vitro;* and the inhibitory action of lipoids upon the hemotoxins has been demonstrated for pneumococcus lysin by Cole (8).

Reduction of Inactive Oxidized Hemotoxin Present in Solutions of the "Protein Fraction" of Pneumococcus Cells.

The hemotoxin of Pneumococcus, unlike most bacterial hemotoxins, is endocellular. It is apparently protein in nature, possesses the power of inducing antibody formation, and is destroyed by trypsin digestion (Cole (8)). All this being true, it seemed probable that the

hemotoxin might be included in the "protein" fraction of the Pneumococcus which is precipitated by acetic acid in the cold (16). A solution of the "pneumococcus protein" furnished by Dr. Avery was used in the following experiment.

This solution had been prepared from Strain F, a Type IV Pneumococcus, by the method described by Avery and Heidelberger (16). It contained 2.8 mg. of protein in 1 cc., had not been protected from air, and was several weeks old when used.

Preliminary tests showed that the solution was non-hemolytic when an amount as large as 0.3 cc. was added to 0.5 cc. of red blood cells. This indicated that if the hemotoxin had been precipitated by the acid, it was present in the inactive form.

Reduction tests similar to those described in experiments with the oxidized "pneumococcus extracts" were made. Two portions of 0.5 cc. of the protein solution were placed in separate tubes; to one of the tubes, 0.5 cc. of 0.1M phosphate solution was added; 0.5 cc. of 0.4 per cent solution of hydrosulfite was added to the other tube. Both tubes were sealed. At the end of the 15 minute period which was allowed for reduction, portions of the "reduced" solution and of the phosphate-diluted control were added to 0.5 cc. of red blood cells. The volume of each hemolysis test mixture was adjusted to 1.0 cc. by the addition of salt solution. After 1 hour's incubation at 38°C., the tests were centrifuged and observations made of the hemolysis.

Samples as large as 0.6 cc. of the phosphate-diluted control gave no hemolysis, which confirmed the previous observation of the absence of active lysin in the protein solution. On the other hand, in the case of the solution treated with the reducing agent, samples as small as 0.03 cc. gave complete hemolysis.

The results of this experiment indicate that the pneumococcus hemotoxin is precipitated by acetic acid in the cold, and is included in solutions of the "protein fraction" of the cell. Although the hemotoxin is not oxidized rapidly in solutions of washed cells in the absence of other easily oxidized substances (4), it is probably slowly oxidized during the preparation and storage of the protein solutions. There is every reason to believe that the inactive lysin which was "reactivated" in the above experiment is identical with the reversible oxidation product of pneumococcus hemotoxin which has been reported in the preceding experiments with sterile pneumococcus extracts.

Reversibility of the Oxidation-Reduction of Pneumococcus Hemotoxin.

The endocellular nature of pneumococcus hemotoxin, which is not liberated into the culture fluid until disruption of the cell membrane, made possible the fol-

lowing experiment which would be difficult to attempt with "exocellular" bacterial lysins set free during the stage of active growth of the culture.

The object of the experiment was to determine whether both the oxidation and reduction processes can be induced by bacterial cells of the sort from which the hemotoxin was originally derived. Pneumococci possess both oxidizing and reducing powers (1, 10, 13), the reaction induced being dependent upon the presence or absence of air. Hence, pneumococci can be employed as the agents for both oxidation and reduction processes, by admitting or excluding air. If only young, unautolyzed cells are used, it is possible to remove the bacteria by centrifugation and obtain a supernatant solution which includes no hemolytic substances introduced by the microorganisms used as oxidizing and reducing agents. This point was carefully controlled by parallel tests carried out with mixtures of broth and pneumococci at the time of each reduction and oxidation treatment.

The experiment consisted in several successive reversible oxidations and reductions of the hemotoxin by young, unautolyzed pneumococci. The oxidation in each instance was effected by aeration of the mixture of hemotoxin solution and bacterial cells. After allowing time for the oxidation of the hemotoxin, the bacteria were removed by centrifugation, and the supernatant solution was "titrated" for active hemotoxin. The oxidized solution was then again reduced by adding a fresh suspension of young pneumococci and sealing the reduction mixture from air. When sufficient time had elapsed for reduction, the mixture was centrifuged again to remove the bacteria, and the supernatant of the reduced solution was titrated for active hemotoxin content.

This procedure was repeated with the same original solution of hemotoxin for three successive series of alternate oxidations and reductions. The sequence of conversions of the same solution of hemotoxin from the active reduced form to the inactive oxidized form, and back again, is outlined in Table V.

Solution I represents the original active hemotoxin present in the reduced extract at the beginning of the experiment. The first oxidation of the hemotoxin was effected by the oxidizing agents formed in the extract itself when exposed to air (4). The oxidized extract, owing to the inactivation of the labile cellular components of its oxidation-reduction systems, is no longer able to oxidize hemoglobin or reduce methylene blue and methemoglobin (11). Since the extract itself is rendered devoid of oxidizing and reducing activity after the first oxidation treatment, Solutions II, IV, and VI can be regarded simply as solutions of oxidized hemotoxin. Thus, the successive oxidations (inactivations) and reductions (reactivations) in Solutions II to VI can be referred directly to the oxidizing or reducing action of the young pneumococci added to the respective mixtures.

TABLE V.

The Reversible Oxidation and Reduction of Pneumococcus Hemotoxin by Living Pneumococcus Cells.

Oxidation or reduction mixture.	Treatment.	Oxidizing or reducing agent.	Period of oxidation or reduction.	Hemolytic activity. Amounts of mixture expressed in terms of original extract.				
				0.06 cc.	0.03 cc.	0.02 cc.	0.01 cc.	0.006 cc.
Solution I	Original solution.	Sterile pneumococcus extract sealed from air.		++++	++++	++++	++++	+++
Solution II	Solution I after oxidation.	Sterile pneumococcus extract in the presence of air.	1.5 hrs.	−	−	−	−	−
Solution III	Solution II after reduction.	Young, intact pneumococci in the absence of air.	2.5 "	++++	++++	+++	+	−
Solution IV	Solution III after oxidation.	Young, intact pneumococci in the presence of air.	1.0 hr.	−	−	−	−	−
Solution V	Solution IV after reduction.	Young, intact pneumococci in the absence of air.	3.0 hrs.	++++	+++	++	±	−
Solution VI	Solution V after oxidation.	Young, intact pneumococci in the presence of air.	0.5 hr.	−	−	−	−	−
Solution VII	Solution VI after reduction.	$Na_2S_2O_4$ in the absence of air.	10 min.	++++	++++	++++	++	+
Supernatant of controls (broth plus young pneumococci) for each reduction and oxidation mixture.				−	−	−	−	−

Control Tests with Mixtures of Broth and Pneumococci.—Broth was treated in exactly the same manner as the solutions of the hemotoxin. Upon removal of the bacteria by centrifugation after each oxidation and reduction period, the supernatant of these mixtures was tested for hemolytic activity. They never contained a trace of hemolytic substances, a fact which can be taken as proof that no hemotoxin was liberated by the cells used in the experiment proper.

The results of this experiment (Table V) illustrate the reversible oxidation-reduction of pneumococcus hemotoxin by pneumococcus cells. This experiment is analogous to one upon the reversible oxidation-reduction of blood pigment by pneumococci (Table II, in a previous paper (14)). Here, too, longer periods of time were required for the anaerobic reduction of the hemotoxin than for its oxidation in the presence of air.

COMMENT.

Pneumococcus hemotoxin is an actual constituent of the bacterial cell. In the reduced condition it possesses certain properties which are lost upon oxidation. As it exists in the cell it is protected by the reducing activity of the living bacteria. If the cells are sufficiently exposed to air, oxidizing agents are formed which oxidize the hemotoxin. If, however, the exposure to air is not too prolonged, nor sufficiently drastic to inactivate completely the reducing activity of the cells, this cellular constituent can be reconverted to the original reduced substance whenever the tension of molecular oxygen tension does not mask the native reducing powers of the bacterial cells. The failure of the reduction systems present in the sterile filtered extracts to "reactivate" subsequently the oxidized hemotoxin in aerated extracts is analogous to the loss of their ability to reduce methylene blue and methemoglobin. As previously shown, the loss in reducing power is due to the inactivation of the thermolabile constituent of the oxidation-reduction system (11). Apparently, these thermolabile constituents are more readily inactivated or "overtaxed" in the sterile, filtered, cell extracts than in the living intact bacterial cell.

While the hemotoxin itself may be of no physiological importance, it is highly probable that the reversible oxidation-reduction of some other thermolabile cellular constituents is involved in the maintenance of the physiological activity of bacterial cells.

SUMMARY.

Pneumococcus hemotoxin, an antigenic substance of bacterial origin, is converted by oxidation to a product devoid of hemolytic action. The oxidation product of the hemotoxin may be converted to the original hemolytic substance by reduction, by the anaerobic action of certain bacteria, or by sodium hydrosulfite. The active lysin, or hemotoxin, produced by the reduction of the inactive oxidized extracts is identical with the original, active, reduced hemotoxin; it possesses the same degree of thermolability and is neutralized by the same specific antibody. The inactive products formed by heating the hemotoxin anaerobically cannot be "reactivated" by reducing agents. The immunological significance of these relations will be discussed in a subsequent paper.

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STUDIES ON THE OXIDATION AND REDUCTION OF IMMUNOLOGICAL SUBSTANCES.

II. The Hemotoxin of the Welch Bacillus.

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INTRODUCTION.

The preceding paper reported a study of the oxidation-reduction of pneumococcus hemotoxin. Another hemotoxin, the lysin of the Welch bacillus, has been used as the subject of the present study. In contrast to the pneumococcus hemotoxin, which is endocellular and an actual constituent of the bacterial cell, the Welch lysin is an "exocellular" substance which is liberated into the culture fluid prior to cell disintegration.

The early work of Welch and Nuttall (2), Welch and Flexner (3), and Herter (4) established the existence and suggested the importance of the Welch bacillus. The studies of Bull (5) and of Bull and Pritchett (6) proved the existence of a true toxin in cultures of this organism. While the present paper is concerned only with the hemolytic substance, the hemotoxin, it is desirable to differentiate this lysin from the mixture of other toxic substances which may be contained in culture fluids of the Welch bacillus. Kojima (7) reports three toxic substances: the hemotoxin, a "false" toxin, and a true toxin. The "false" toxin and the true toxin seemed to be quite distinct, the hemotoxin and the true killing toxin were less sharply differentiated, although the hemotoxin proved to be somewhat more thermolabile. Henry (8, 9) distinguishes between the hemotoxin and the true killing toxin which he terms "myotoxin" and attempted to separate the two substances by selective absorption.

The occurrence of the lysin and its properties have been studied by a number of workers (4, 9, 19). Most authorities agree that it is thermolabile; it is non-dialyzable and may be precipitated by alcohol, ammonium sulfate, and other protein precipitants. It is inhibited or neutralized to some extent by normal serum from different animals (16, 20), at least a part of this action probably being

attributable to lipoidal serum constituents. A wide variation may occur in the inhibitory action of the sera of different, normal rabbits (20).

The hemotoxin of the Welch bacillus is reported to be a true antigen, inciting an immunity response when injected into animals. The production of a specific antihemotoxin neutralizing the hemotoxin has been reported by Schlossberger (21), Wuth (22), and others. The specificity is apparently a "species specificity;" the hemotoxin is not neutralized by sera produced by immunization with other anaerobic bacteria such as the gas edema bacillus (22), nor does the Welch antihemotoxin neutralize the hemotoxin of the tetanus bacillus. Studies on the neutralization of the hemotoxin by the immune antihemotoxin have been made by Henry (9).

EXPERIMENTAL.

Methods.

The methods used in the following experiments are essentially the same as those employed in the previous study (1). Well washed sheep cells were used in the hemolysis tests. Approximately constant concentrations of cells were obtained by colorimetric standardization of the stock suspensions from which the dilutions were made.

The three strains of the Welch bacillus used in the experiments were isolated from heated stools by Dr. Robert Nye of the Boston City Hospital. The cultures were grown in broth, previously boiled and sealed with a heavy layer of vaseline. Infusion broth yielded more active fluids than did meat extract broth. The culture fluids used as the source of the hemotoxin were not filtered but were rendered free from bacterial cells by prolonged centrifugation.

The Influence of the pH of the Hemolysis Test System upon the Activity of Welch Lysin.

It was necessary first to determine the influence of the pH of the medium in which the blood cells are suspended. For this purpose, comparisons were made of the activity of the lysin upon cells suspended in physiological salt, with its activity upon cells suspended in 0.1 M phosphate solutions ranging from pH 7.0 to 8.0. The following tests were made: (1) "titrations" of the smallest amount of lysin required for the hemolysis of cells suspended in the different solutions; (2) comparisons of the time required for hemolysis of a constant volume of cells by a constant amount of lysin; (3) comparisons of the degree of hemolysis effected by a constant amount of lysin in a constant time. Protocols illustrating the results of these experiments are given in Table I.

It is evident (Table I) that the Welch lysin is much less active when the blood cells are suspended in phosphate solutions of pH 7.5 to 8.0, than if the cells are suspended in salt solution or in pH 7.0 phosphate.

It now follows that comparisons of the lysin content of culture fluids of different pH will be valid only if precautions are taken to maintain a constant pH in the hemolysis test systems. Hence, in all of the following experiments, the blood cells were suspended in buffer mixtures and control tests were made to prove that the acidity or alkalin-

TABLE I.

Comparative Influence of Phosphate Mixtures of Different pH upon the Activities of Welch Lysin and Tetanolysin.

(*a*) "Titration" of the lysins.

Welch lysin.					Tetanolysin.				
	Hemolysis of blood cells suspended in					Hemolysis of blood cells suspended in			
Amount of lysin.	NaCl	pH 7.0 phosphate.	pH 7.5 phosphate.	pH 8.0 phosphate.	Amount of lysin.	NaCl	pH 7.0 phosphate.	pH 7.5 phosphate.	pH 8.0 phosphate.
cc.					*cc.*				
0.03	++++	++++	++++	−	0.009	++++	++++	++++	++++
0.02	+++	++++	++	−	0.006	++++	++++	++++	+++
0.01	+	+++	−	−	0.003	++	+++	++	++

(*b*) Comparison of relative degree of hemolysis by a constant amount of lysin in 1 hr. at 38°C.

Blood cells suspended in	Relative degree of hemolysis.	
	Welch lysin.	Tetanolysin.
	*per cent**	*per cent**
NaCl	90–95	70–75
pH 7.0 PO_4	100	100
pH 7.5 PO_4	20	90–95
pH 8.0 PO_4	Less than 10.	75–80

* The per cent hemolysis presented in the table is relative and is calculated by considering hemolysis in the pH 7.0 phosphate as 100 per cent.

ity of the fluid added in the lysin tests was compensated by the buffered suspension medium.

If the differences in the hemolytic activity of Welch lysin in the different solutions in which the blood cells were suspended were due simply to some effect which rendered the blood cells more "fragile," one would expect other bacterial lysins to show the same marked differ-

ences at different pH. To show that this is not the case, a comparison of Welch lysin and tetanolysin is also presented in Table I. It is seen there that, while affected to some extent by the pH of the medium in which the cells are suspended, tetanolysin does not show the marked differences in activity which are exhibited by Welch lysin. This also seems to rule out any difference which might be due to slight differences in tonicity of the buffer mixtures at different pH or to effects due to shifts in the ratio of potassium and sodium in the different buffer mixtures.

This influence of pH upon the activity of Welch lysin is of importance in two other connections. First, the pH of the circulating blood is not the optimum reaction for hemolysis by Welch lysin and this in itself, together with the inhibitory action of normal serum, offers a certain degree of protection against *in vivo* hemolysis. Second, many of the early reports that "neutralized" Welch culture fluids were devoid of lytic action may be explained by the fact that fluids "neutralized" to phenolphthalein (above pH 8.5) are too alkaline to offer a fair test for the activity of Welch lysin.

Heat Lability of Welch Lysin.

In most of the experiments on the heat lability of Welch lysin the fluids heated were at pH 6.5; the heating tests were made in vaseline-sealed tubes. It was found that the lysin is destroyed rapidly at 55°C., since 10 minutes at this temperature suffices for its complete destruction. These results indicated a somewhat higher degree of lability than that reported by the majority of the previous workers (4, 9, 11, 14, 16). In view of the effect of pH upon heat inactivation, further experiments were made with the lysins of three strains adjusted to pH ranging from 3.0 to 9.0. All of these were inactivated by 10 minutes exposure to 60°C. (no tests were made at 55°C.).

Inactivation of Welch Hemotoxin by Exposure to Air and "Reactivation" of the Oxidized Lysin by Treatment with Reducing Agents.

A number of preliminary experiments showed that actively hemolytic fluids of Welch bacillus cultures lost their hemolytic power after exposure to air in Erlenmeyer flasks at room temperature. Since the hemotoxin proved perfectly stable for months in sealed tubes, it seemed probable that the inactivation process was an oxidation.

Tests were next made to determine if the inactive product of the Welch lysin, like the inactive oxidation product of *pneumococcus* hemotoxin, can be converted to the original, actively hemolytic substance by the action of reducing agents. These experiments were performed in the manner described in the preceding paper.

The protocol of a typical experiment is given in Table II.

The results given in Table II prove that Welch lysin which has been inactivated by aeration can be "reactivated" by the reducing action of bacteria or of sodium hydrosulfite. Hence, as previously demonstrated for *pneumococcus* hemotoxin, aeration of Welch bacillus culture fluids yields a reversible oxidation product which can be converted to the originally active substance by reduction treatment.

TABLE II.

"Reactivation" of Oxidized Welch Lysin by Treatment with Reducing Agents.

	Hemolytic activity.		
	Amount of culture fluid.		
	0.1 cc.	0.03 cc.	0.01 cc.
Oxidized lysin (untreated)	–	–	–
Oxidized lysin treated with hydrosulfite	++++	++++	++++
Oxidized lysin treated with non-hemolytic anaerobic bacilli	++++	++++	++++

Attempts to "Reactivate" Welch Lysin Which Has Been Inactivated by Heat.

Experiments analogous to those described in the preceding paper were also made to determine if heat-inactivated Welch lysin can be "reactivated" by reduction. The results of these experiments were the same as those with pneumococcus hemotoxin; heat-inactivated Welch lysin cannot be "reactivated" by the action of reducing agents. This fact demonstrates that the products formed when Welch lysin is inactivated by heat are distinctly different from the inactive products formed when the lysin is exposed to air.

Comparison of the Heat Lability of Reduced (Active) Welch Lysin with the Lability of the Oxidized (Inactive) Lysin.

The following experiment compares the degree of heat resistance possessed by the oxidized and the reduced lysin. The heating treatments were chosen from the data of preliminary experiments, so that one series was heated a period (10 minutes at 55°C.) just sufficient to destroy completely the reduced hemotoxin, and the other series was heated a period (2½ minutes at 55°C.) which had been found sufficient to inactivate somewhat over half of the reduced hemotoxin. Both the reduced and the oxidized lysin were heated in vaseline-sealed tubes. The protocol of this experiment is given in Table III.

TABLE III.

Comparison of the Heat Lability of Reduced and Oxidized Welch Lysin.

Amount of lysin.	Unheated.			Heated 2½ min. at 55°C.			Heated 10 min. at 55°C.		
		Oxidized lysin.			Oxidized lysin.			Oxidized lysin.	
	Reduced lysin.	Not treated with reducing agents.	After treatment with hydrosulfite.	Reduced lysin.	Not treated with reducing agents.	After treatment with hydrosulfite.	Reduced lysin.	Not treated with reducing agents.	After treatment with hydrosulfite.
cc.									
0.2	++++	−	++++	++++	−	++++	−	−	−
0.1	++++	−	++++	++++	−	+++	−	−	−
0.05	++++	−	++++	+	−	±	−	−	−
0.02	++++	−	++++	−	−	−	−	−	−
0.01	++++	−	++++	−	−	−	−	−	−

The data presented in Table III show that the oxidized and reduced Welch lysin possess identical heat resistances. Not only are both destroyed entirely by 10 minutes exposure to 55°C., but the rate of destruction of the two substances is apparently the same, since approximately equal amounts of the oxidized and the reduced substance remain undestroyed after 2½ minutes heating. In view of the important change undergone by the lysin molecule when it is oxidized, the retention of its original degree of heat resistance is remarkable.

Influence of pH upon the Oxidation of Welch Lysin.

The following experiment is concerned with the effect of pH upon the subsequent stability of hemotoxin previously formed in broth of

the optimum pH for lysin production. Cultures of the Welch bacillus were grown in poorly buffered infusion broth the initial reaction of which was approximately neutral. Two series of the lysin-containing broth were then adjusted to different pH by the addition of sterile acid and alkali. One series was protected from air by a heavy vaseline seal; the other series was freely exposed to air. At different periods of time, the stability of the hemotoxin in both the sealed and aerated series was compared by titrations of the hemolytic activity.

In the lysin titrations themselves each hemolytic test system was at approximately the same pH. Control tests were made to prove that the addition of each amount of lysin used did not change the pH of 2.5 cc. of 0.1 M phosphate solution (pH 7.0). Only the larger amounts of the more acid and alkaline lysins caused any change in pH; these cases were controlled by substituting pH 6.8 or pH 7.2 phosphate solution in the indicated instances.

The lysin was added directly to 2.5 cc. of 0.1 M phosphate solution (pH 7.0); after mixing the lysin and the buffer solution 1 drop of a 40 per cent suspension of blood cells was added. The hemolytic test mixtures were incubated 1 hour at 38°C. and then centrifuged.

Considerable precipitation occurred in the acidified lysin, especially in the sealed tubes. As far as was possible, this precipitate was avoided and the clear supernatant was taken for the samples used in the hemolytic tests. The pH was determined colorimetrically at the time of the 13 hour tests.

The results of the tests of the series in sealed tubes showed that Welch lysin retains its activity for at least 24 hours within a pH range of 6.0 to 9.0, provided air is excluded. This fact indicates that differences in the rate of inactivation of the lysin in the aerated series during the period of the experiment can be referred to the effect of pH upon oxidation of the lysin. The results of the tests of the aerated series are presented in Table IV.

To avoid the complications introduced by acid precipitation of the lysin, it is advisable to limit the analysis of Table IV to a comparison of the rate of inactivation of the lysin in the fluids adjusted to pH 6.0, 7.2, and 8.8. In the pH 8.8 fluid, most of the lysin was inactivated after only 4 hours exposure to air. All active lysin had disappeared in the pH 7.2 and pH 8.8 fluids in less than 24 hours, although traces of active lysin persisted in all of the more acid fluids for over 48 hours. The lysin proved most stable in the fluid adjusted to about pH 6.0. From these results it is apparent that within the range of pH 6.0 to

TABLE IV.

Influence of pH upon the Oxidation of Welch Lysin.

Amount of fluid.	Original lysin, before exposure to air.	Hemolytic activity of centrifuged culture fluid adjusted to different pH, after aeration. Lysin adjusted to pH 8.8; after exposure to air for			Unadjusted lysin (pH 7.2)*; after exposure to air for		Lysin adjusted to pH 6.0; after exposure to air for		Lysin adjusted to pH 5.5; after exposure to air for		Lysin adjusted to pH 4.5; after exposure to air for	
		4 hrs.	9 hrs.	24 hrs.	9 hrs.	24 hrs.	9 hrs.	24 hrs.	9 hrs.	24 hrs.	9 hrs.	24 hrs.
cc.												
0.20	++++	++++	−	−	++++	−	++++	++++	++++	++	++++	++
0.10	++++	+++	−	−	++++	−	++++	++++	++++	−	++++	−
0.05	++++	+	−	−	++++	−	++++	+++	++++	−	+++	−
0.04	++++	−	−	−	+++	−	++++	+++	++++	−	++	−
0.03	++++	−	−	−	+	−	++++	++	++++	−	+	−
0.02	++++	−	−	−	−	−	++++	−	+++	−	−	−
0.01	++++	−	−	−	−	−	+++	−	+	−	−	−
0.005	+++	−	−	−	−	−	+	−	−	−	−	−
0.003	+	−	−	−	−	−	−	−	−	−	−	−

* Original pH 6.5, which increased to pH 7.2 after a short period of exposure to air.

9.0, the rate of oxidation of Welch lysin is at least roughly proportional to the alkalinity. On the other hand, in fluids of lower pH, unless precipitated by excessive acidity, the lysin is relatively stable in contrast with its rapid inactivation when exposed to air in slightly alkaline solutions.

The unadjusted culture fluid becomes more alkaline when exposed to air; and at least a part of the rapid change from approximately pH 6.5 to pH 7.2 can be explained by the loss of carbon dioxide. Since the lysin is oxidized more rapidly in alkaline solutions, the considerable increase in pH which occurs when the culture fluid is exposed to air must accelerate the inactivation of the lysin. In that sense, the processes involved in the inactivation of the Welch hemotoxin in aerated solutions can be considered as autocatalytic.

Detection of the Presence of Inactive Hemotoxin in Culture Fluids Containing No "Active" Lysin.

A number of 2 months old cultures of Welch bacilli were examined in the usual way for the presence of active lysin. The vaseline seals of some of the cultures had been blown off by the vigorous gas formation; others, with intact seals, were in broth more alkaline than pH 7.5. None of these cultures showed the presence of any active lytic substance in tests with as much as 1.0 cc. of the fluid. However, after the bacteria-free fluids were treated with the reducing agent, amounts as small as 0.02 cc. gave complete hemolysis.

These results are easily explained, of course, by the preceding experiments. They show, however, the possibility of demonstrating the presence of oxidized lysin in culture fluids which would be pronounced free of hemotoxin if examined in the ordinary way. It would seem desirable to apply such tests to all culture fluids of unknown origin before pronouncing the tested strain to be devoid of the power of producing an active lysin.

DISCUSSION.

The hemotoxin of the Welch bacillus is inactivated by exposure to air. The inactivation is an oxidation process and is reversible. The reversal can be brought about by biological agents or by the chemical agent, sodium hydrosulfite, the same agents that is to say, which served

in the reduction of the reversible oxidation product of hemoglobin and of pneumococcus hemotoxin. On the other hand, the heating of the hemotoxin yields "inactive" products which cannot be "reactivated" by reduction treatment.

The active reduced form and the inactive oxidized form of the lysin show no difference in the irreversible change brought about by heat. It seems logical to assume that the heat inactivation is in the nature of a protein denaturization. On the other hand, the marked change induced in the lysin by oxidation must be of entirely different nature and similar to those involved in the conversion of hemoglobin to its reversible oxidation product, methemoglobin.

The rate of oxidation of Welch lysin is influenced by the pH of the culture fluid. If the lysin is exposed to air in fluids adjusted to approximately pH 6.0, the oxidation proceeds much more slowly than in systems more alkaline than pH 7.0. In broth adjusted to more acid zones, around pH 4.0 and pH 5.0, the lysin is more stable than when aerated in alkaline broth, but much of the lysin is precipitated in the more acid systems. In considering the influence of pH upon oxidation of the lysin, it must be remembered that the hemotoxin of the Welch bacillus, like the lysin of Pneumococcus, may be oxidized, not by the direct action of molecular oxygen, but by oxidizing agents formed during the oxidation of other substances in the culture fluid. If this is the case, the observed influence of pH may be due not to the direct effect of pH upon the ease of oxidation of the lysin itself, but to its effect upon the formation of oxidizing agents from other substances.

It is probable that the gradual deterioration of Welch lysins which occurs in culture fluids stored under usual laboratory conditions is due in large part to oxidation processes. Where the "deterioration" really is an oxidation, the conditions determining the ease of oxidation of the lysin will determine its stability. Among these conditions, pH has been shown to be very important, and it is probable that the pH of the fluids explains the relatively high degree of stability of the Welch lysin in our broth cultures. When stored at room temperature under vaseline seal in broth cultures with a final pH of approximately 6.4, the lysin activity remained practically unchanged for months. (Since the Welch bacillus is not a strongly proteolytic organism, the hemotoxin, which is probably protein in nature, is not apt to be inactivated by

proteolytic hydrolyses.) On the other hand, in culture fluids of final pH 7.5, the lysin gradually deteriorated after the 2nd week's storage at 37°C. and was completely inactive after 3 weeks. This occurred in cultures under a heavy seal of vaseline and under conditions identical in all respects, except that of pH, with those of the cultures at pH 6.2 which retained their original lysin activity. Since the inactive product in these sealed tubes of the pH 7.5 fluids was "reactivated" by treatment with hydrosulfite, it is evident that the observed "deterioration" of the lysin was an oxidation. While traces of oxygen may have passed through the seal, it would seem that the reducing action of the broth itself would be sufficient to compensate for these traces of air. Possibly, in alkaline systems the direct participation of oxygen is not required in the oxidation of the Welch lysin. At any event, it appears that in alkaline systems (as pH 7.5), the "active" reduced lysin is slowly converted to its "inactive" oxidation product even though the system possesses sufficient reducing activity to prevent the conversion of methylene white to methylene blue.

If the Welch lysin be considered as an antigen of bacterial origin the results of the above investigation are of considerable immunological interest from various theoretical points of view. Among the most suggestive relations illustrated are the following: the reversible oxidation and reduction of an antigenic substance; the inability of the oxidation product to injure (hemolyze) the cell; and the marked influence of pH upon the oxidation of the hemotoxin. These relations suggest a number of potential applications to more important antigens, which it is fruitless to discuss until immunological evidence has been accumulated.

SUMMARY.

A study has been made of the oxidation and reduction of the hemotoxin of the Welch bacillus. The oxidation of the lysin results in the loss of its hemolytic activity. The "inactive" oxidation product of the Welch lysin may be reversed to the "active" lysin by treatment with biological or chemical reducing agents. The lysin, both in its reduced and oxidized form, is irreversibly inactivated by the same heat treatment. The pH of the system is an important factor in the oxidation of the lysin, the process proceeding much more rapidly at a pH above 7.0.

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STUDIES ON THE OXIDATION AND REDUCTION OF IMMUNOLOGICAL SUBSTANCES.

III. Tetanolysin.

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In the preceding papers, certain principles previously established in studies of the oxidation-reduction of blood pigments have been applied to the oxidation-reduction of pneumococcus hemotoxin (1) and the lysin of the Welch bacillus (2). In the present paper, these studies are extended to tetanolysin, the hemotoxin produced by the tetanus bacillus.

The hemotoxin, or lysin, of the tetanus bacillus was first recognized by Ehrlich (3, 4) who clearly differentiated it from the true killing toxin. Much of the lysin-inhibiting or lysin-neutralizing effect of normal sera (3–7) can be referred to the lipoid constituents (8, 9, 10, 13) although several investigators (11, 12) have reported that certain protein constituents are also concerned.

The antibodies of tetanolysin and tetanospasmin have been differentiated by later workers on much the same grounds as those of Ehrlich. Further evidence of the specificity of the tetanus hemotoxin is furnished by the fact that it is not neutralized by the antihemotoxin produced by immunization with the lysins of Pneumococcus,[1] staphylococcus (14), and of other, anaerobic bacteria (15).

Tetanolysin has been used to illustrate the rate of combination of toxins and antitoxins, in so called "*Heilversuche*" experiments (16, 17); to demonstrate the effect of fractional addition of toxins in toxin-antitoxin mixtures (19); and in a number of other fundamental investigations. The physical-chemical relations involved in the reactions by which the toxin is destroyed, and the effect of temperature upon the rate of hemolysis have also been studied (4, 19, 20, 21, 10). As shown in the preceding paper (2) the activity of tetanolysin is influenced to a much less marked degree by differences in the pH of the hemolysis test system, than is the Welch lysin.

[1] Unpublished experiments of our own.

Tetanolysin has been chosen for the purposes of this investigation, as an example of an antigen of bacterial origin. This lysin has more than ordinary interest, since it has been used in classical experiments, the results of which have been applied in the establishment of many of the fundamental principles of immunology. The experiments reported here are concerned with the conditions governing the oxidation-reduction of tetanus hemotoxin (tetanolysin), and with the nature of the hemolytically inactive products formed during the oxidation of the active lysin.

EXPERIMENTAL.

Methods.

The methods used in this investigation were essentially the same employed in the preceding studies (1, 2). A typical strain of tetanus bacillus, which was furnished by Dr. Charles Krumwiede of the Bureau of Laboratories of the New York City Department of Health, was used in all of the experiments.

In measuring the lysin content of culture fluids treated with hydrosulfite, it is important to avoid exposure of the "reduction mixture" to air before its addition to the red blood cell. The possibility of the destruction of the lysin by neglect of this factor was pointed out in the first paper (1).

In later experiments, the following procedure has proved satisfactory. The mixtures of lysin fluid plus reducing agent were sealed with vaseline as previously described. After a reduction period of 10 to 15 minutes, measured amounts of the reduced fluids are added immediately to the blood cell solution used in the final hemolysis tests. The blood cells are held in a cold water bath at 8–10°C. for 15 to 20 minutes before being placed at 38°C. The test mixtures are shaken several times during the preliminary incubation period at low temperature. This procedure has proved more satisfactory than incubating them immediately at 38°C. Comparative lysin titrations of unoxidized fluids not treated with hydrosulfite with the same fluids treated with the reducing agent have emphasized the importance both of rapid addition of the test samples to the blood cells, and of the preliminary period at low temperature.

If the hydrosulfite-treated fluids are exposed to air for even a few minutes, some of the lysin is destroyed; if the aeration is prolonged for an hour and a half, practically all of the lysin may be destroyed. This rapid destruction of the lysin is undoubtedly due to agents formed from the hydrosulfite in presence of air, since the mixtures of lysin fluid plus phosphate solution are not rapidly destroyed when exposed to air.

Relations very similar to the above were encountered by Conant and Fieser (25) in the determination of hemoglobin in solutions previously reduced by hydro-

sulfite. They were also evident in earlier experiments of our own (26) on the determination of hemoglobin in mixtures previously reduced by bacteria in the absence of air. The advantage of low temperature in the preliminary incubation of the hemolysis tests probably depends upon the same principles that made it more desirable to saturate the hemoglobin at low temperature. The lysin can combine with the blood cell at a low temperature just as hemoglobin can combine with oxygen, although the destruction of both the lysin and the hemoglobin by the products of hydrosulfite is retarded to a greater extent by decreased temperature. Possibly also, the lysin after it once combines with the blood cell is less easily oxidized or destroyed, just as ferrous hemoglobin proves more stable after combination with oxygen or carbon monoxide (26).

TABLE I.

Tetanolysin Accumulation in Plain Broth and Glucose Broth at Different Ages of the Culture.

Amount of culture fluid.	Plain broth culture.			Glucose broth culture.		
	Age of culture.			Age of culture.		
	16 hrs.	36 hrs.	12 days.	16 hrs.	36 hrs.	12 days.
cc.						
0.03	++++	++++	++++	++++	++++	++++
0.01	++++	++++	++++	++++	++++	++++
0.004	++	++	+++	+++	++++	++++
0.002	−	−	+	+	+	+
0.001	−	−	−	±	±	±

Tetanolysin Accumulation in Plain Broth and Glucose Broth at Different Ages of the Culture.

The primary object of the following experiment was to determine the period of growth of the culture at which the lysin is produced. It was also desired to determine the stability of the lysin when cultures were held at 38°C. under the usual conditions.

The cultures were grown in infusion broth containing no added glucose and in the same broth containing 1 per cent glucose. The lysin was added to 3 cc. of 2 per cent sheep cells suspended in salt solution; the hemolytic test mixtures were incubated 1 hour at 38°C. and then centrifuged. The results are given in Table I.

The results of these experiments (Table 1) show that tetanolysin, like the Welch lysin, is produced early in the growth of the culture. This fact distinguishes both of these lysins from the endocellular hemotoxin of Pneumococcus

which is liberated in the medium only upon disintegration of the bacterial cells.

The lysin content of both the plain broth and the glucose broth cultures remained surprisingly constant in cultures varying in age from 16 hours to 12 days. (The pH of the cultures, if measured with a minimum exposure to air, was approximately 6.5.) The presence of glucose influenced the lysin production to a very slight degree; though when any difference existed, it was in favor of the glucose broth culture. In similar experiments with the Welch bacillus a greater lysin content was obtained in the absence of glucose. One would naturally expect the presence of glucose to alter the products of growth of the gas bacillus to a greater extent than it would in the case of the tetanus organism. However, the lower yield of lysin in glucose cultures of the gas bacillus cannot be referred entirely to the influence of the presence of the fermentable sugar upon the direction of the metabolism of the bacteria. These cultures become quite acid and the precipitation of the lysin or other effects of acidity must also be considered as factors.

TABLE II.

Heat Lability of Tetanolysin.

Amount of lysin.	Original lysin (titre).	Hemolysis by lysin heated for			
		10 min. at 55°C.	10 min. at 60°C.	2½ min. at 65°C.	10 min. at 65°C.
cc.					
0.5	++++	++++	++++	++++	−
0.2	++++	++++	++++	++++	−
0.1	++++	++++	+++	++++	−
0.05	++++	++++	+++	++++	−
0.03	++++	++++	±	+	−
0.02	++++	++++	−	−	−
0.01	++++	++++	−	−	−
0.002	++	±	−	−	−
0.001	±	−	−	−	−

Heat Lability of Tetanolysin.

Experiments were made to compare the lability of the tetanus hemotoxin with the lability of the other hemotoxins studied in this series of papers. The lysin was heated in narrow thin walled tubes protected from air. The reaction of the fluids tested, if not exposed to air, was pH 6.6. The tests for active lysin were made immediately after the heating test. The results are given in Table II.

The results of these experiments (Table II) show that tetanolysin is completely inactivated by 10 minutes exposure to 65°C. At this temperature the inactiva-

tion processes proceed rapidly, as about the same amount of lysin is destroyed by 2½ minutes exposure to 65°C. as is destroyed in 10 minutes at 60°C. This degree of heat resistance is considerably higher than that found in tests made under similar conditions with the hemotoxins of the Pneumococcus, the Welch bacillus, and El Tor strains of the cholera vibrio. The order of heat lability of the hemotoxins of these different bacteria is as follows: Pneumococcus, cholera vibrio, Welch bacillus, and tetanus bacillus.

Inactivation of Tetanolysin by Exposure to Air, and "Reactivation" of the Oxidized Lysin by Treatment with Reducing Agents.

Experiments were made to determine whether the inactive substances formed in aerated tetanus culture fluids included a reversible oxidation product. The methods employed were the same as those

TABLE III.

"Reactivation" of Oxidized Tetanolysin by Reduction.

Tetanolysin.	Hemolytic titre. Amount of lysin.			
	0.10 cc.	0.03 cc.	0.01 cc.	0.005 cc.
Oxidized lysin not treated with $Na_2S_2O_4$.	−	−	−	−
Oxidized lysin after reduction by $Na_2S_2O_4$	++++	++++	++++	++

used in the preceding investigations. A protocol of a typical experiment is given in Table III to illustrate the results.

The "reactivation" of the hemolytically inactive fluid by treatment with the reducing agent (Table III) can be interpreted as the conversion of a non-hemolytic, reversible oxidation product to the originally "active," reduced lysin. Hence, the inactivation of tetanolysin upon aeration of culture fluids is an oxidation process which yields a reversible product.

The Influence of Temperature upon the Oxidation of Tetanolysin.

The following experiments were made to determine the effect of temperature upon the oxidation of the tetanolysin.

The bacteria-free supernatant fluid of a 12 day glucose broth culture was used in the experiments. 10 cc. portions were exposed to air at 5°, 22°, and 38°C. Samples were taken at the times indicated in the protocol. The pH of the cul-

TABLE IV.

Influence of Temperature upon the Oxidation of Tetanolysin.

Amount of culture.	Original (before exposure).	Exposed to air. 5°				Exposed to air. 22°				Exposed to air. 37°			
		2 hrs.	8 hrs.	11 hrs.	24 hrs.	2 hrs.	8 hrs.	11 hrs.	24 hrs.	2 hrs.	8 hrs.	11 hrs.	24 hrs.
cc.													
0.4	++++	++++	++++	++++		++++	++++	++++	++++	++++	++++	+	−
0.2	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++	−	−
0.1	++++	++++	++++	++++	++++	++++	++++	++++	++	++++	±	−	−
0.05	++++	++++	++++	++++	++++	++++	++++	+++	+	++++	−	−	−
0.03	++++	++++	++++	++++	++++	++++	++++	++	−	++++	−	−	−
0.02	++++	++++	++++	++++	++	++++	+	+	−	++++	−		−
0.01	++++	++++	++++	+++	±	++++	−	−	−	++++	−		−
0.004	+++	++	+	++	−	++	−	−	−	++	−		−
0.002	+	+	−	±	−	+	−	−	−	±	−		−
0.001	±	−		−	−								−

ture after exposure to air was 7.2. The hemolysis tests were made with 3 cc. of blood cells suspended in salt solution. The results are given in Table IV.

The data in Table IV illustrate the relative rates of oxidation of tetanolysin at different temperatures. As might be expected, the lysin is oxidized much more rapidly at the temperature of the body than at room temperature, being almost entirely destroyed after 11 hours exposure to air at 37°C. The oxidation proceeds much more slowly at the temperature of the ice box.

Attempt to "Reactivate" Heated Tetanolysin by Treatment with Reducing Agents.

In the preceding papers (1, 2) it has been shown that the heat-inactivated products of the hemotoxins of Pneumococcus and of the Welch bacillus cannot be "reactivated" by reducing agents. This obtained in tests made with lysins which had been heated the minimum amount required for their complete inactivation. Tests were made to determine if the same relation holds true in the case of tetanolysin.

The results of these experiments were the same as those obtained in analogous experiments with the other lysins. If inactivated by heat, neither the "reduced" active lysin nor the "oxidized" lysin can be "reactivated" subsequently by treatment with sodium hydrosulfite. Hence, it would seem that the inactivation of tetanolysin by heat is due to the formation of products distinctly different from the oxidation product.

The Distinction between the Reversible and Irreversible Inactivation of Tetanolysin.

The preceding experiments have dealt with two types of inactivation: that due to oxidation, which yields a reversible inactive product, and that brought about by heat, which is irreversible (protein denaturization). It is possible that when culture fluids are aerated, a part of the lysin may also be converted to irreversible products, just as in mixtures of "ferrous" (24), and "ferric" hemoglobin part of the blood pigment may be changed to irreversible products (globin, hematin, etc.). The detection of the irreversibly inactivated lysin corresponds to that of irreversibly inactivated products of hemoglobin.

In any mixture containing hemoglobin and its degradation products, the amount of "active" hemoglobin can be estimated by determining its oxygen- or carbon monoxide-combining capacity. In the same mixtures, the amount of methemoglobin, the reversible oxidation product, may be estimated by reducing the mixture and then determining the increase in oxygen or carbon monoxide capacity. This increase in "activity" can be referred to the reduction of "inactive" methemoglobin to "active" hemoglobin. The detection of other degradation products, such as globin and hematin, requires data on the initial content of hemoglobin in the original fluid before any deterioration has occurred. With these data obtained (the initial "total hemoglobin"), the further degradation products (such as globin and hematin) are estimated by subtracting the sum of

TABLE V.

Mixtures of "Active" Tetanolysin and Its "Inactive," Reversible Oxidation Product in Culture Fluids Exposed to Air at Different Temperatures.

Amount of culture fluid.	Lysin in untreated fluids (before treatment with reducing agent).				Lysin in fluids after treatment with reducing agents.			
	Unexposed culture fluid.	Culture fluid exposed to air for 24 hrs. at			Unexposed culture fluid.	Culture fluid exposed to air for 24 hrs. at		
		5°C.	22°C.	37°C.		5°C.	22°C.	37°C.
cc.								
0.2	++++	++++	++++	−	++++	++++	++++	++++
0.1	++++	++++	++++	−	++++	++++	++++	++++
0.05	++++	++++	++	−	++++	++++	++++	++++
0.04	++++	++++	−	−	++++	++++	++++	++++
0.03	++++	++++	−	−	++++	++++	++++	+++
0.02	++++	+++	−	−	++++	++++	+++	++
0.01	++++	+	−	−	++++	++++	+++	+
0.005	+++	−	−	−	+++	+++	+	−
0.003	+	−	−	−	++	−	−	−

the hemoglobin and methemoglobin which are present in the deteriorated mixture, from the initial amount of hemoglobin in the undeteriorated fluid. In the case of mixtures of the different modifications of active hemotoxins, the principles involved are exactly the same.

In examination of the lysins, the first data required are measurements of "total lysin." These are obtained by determining the "active lysin" content in undeteriorated culture fluids. The fluid of a young culture, or a fluid which has been protected from deterioration, is titrated for "active lysin" both before and after treatment with a reducing agent. If the lysin content is not increased by the reduction treatment, it is assumed that all of it is present in the "active" form, and that the values obtained represent the "total lysin" produced by the

culture. With the preliminary data at hand, the following measurements are made of the lysin in the deteriorated culture fluids. The "active lysin" is estimated by a titration of the lytic activity of the fluid. The fluid is then treated with the reducing agent and the titration is repeated upon the reduced mixture. As in the case of the blood pigment derivatives, the increase in the "active" substance upon reduction represents the reversible oxidation product. The other degradation products are estimated as the difference between the "total lysin" and the sum of the reduced "active" lysin and the reversible oxidation product.

A number of different mixtures in deteriorated culture fluids have been examined by these methods. The experiment given in Table V represents an examination of the cell-free supernatant of a 12 day culture, portions of which had been exposed to air at 5°, 22°, and 37°C.

The lysin "titrations" of the unexposed culture fluid and of the fluids exposed to air at different temperatures are presented in Table V. The first four columns include the titrations of the fluids prior to treatment with a reducing agent. The results recorded in these columns, therefore, represent the lysin which is in the active or reduced state. The last four columns of the table include the titrations of the same fluids after the reduction treatment. The results of these titrations represent the sum of the active lysin plus the inactive but reversible oxidation product.

A comparison of the lysin activity of the unexposed fluid and of the exposed fluids, before and after treatment with a reducing agent, reveals the following relations. The unexposed fluid exhibits no increase in apparent lysin content upon treatment with a reducing agent, which proves the absence of the reversible oxidation product of the lysin. The value of the lysin titration of the unexposed fluid after the reduction treatment may be taken as "total lysin" present in the culture fluid. In contrast to the unexposed fluid which showed no increase in activity upon reduction treatment, all of the exposed fluids exhibited an unmistakable increase in activity when reduced. The difference between the apparent lysin content of each of the fluids before and after reduction, may be assumed to represent the degree of "reactivation" of the inactive, but reversible oxidation product.

It is evident, however, that only one of the exposed fluids regained the original "total lysin" value after reduction treatment. The failure to regain the original lysin activity, as shown by the dif-

ference between the original or "total lysin" and the sum of the active lysin plus its reversible oxidation product in the deteriorated fluid may most easily be explained by the formation of other irreversible degradation products which probably arise from the deterioration of the first formed reversible oxidation product. The irreversibly inactivated products of the lysin can be compared to degradation products of hemoglobin, such as globin and hematin, which, unlike methemoglobin, cannot be reduced to the original or "active" blood pigment. While this explanation seems the most likely one, it is necessary to admit the possibility of hydrosulfite failing to reduce completely all of the reversible product.

TABLE VI.

Mixtures of Different Products of Tetanolysin in Culture Fluids Exposed to Different Conditions.

Previous treatment of culture fluid.	Ly_r (original, active reduced lysin).	Ly_o (inactive reversible oxidation product).	Ly_x (other inactive degradation products).
Not exposed to air	200	0	0
Exposed to air for 24 hrs. at 5°C.	50	150	0
" " " " 24 " " 22°"	7	143	50
" " " " 24 " " 37°"	0	33	167

It is evident from the conditions of the above experiment that each of the fluids examined (Table V) originally contained the same amount of the active, or reduced, lysin. The various proportions of the different modifications of tetanolysin that may be present in the same culture fluids after exposure under the different conditions can be graphically illustrated by collecting the data of Table V in the form given in Table VI.

The "units" of lysin represent the amount of lysin which suffices to give approximately 80 per cent hemolysis of 4 cc. of a 2 per cent suspension of blood cells. These "units" at best are only approximate and are not presented as representing absolute values. They serve, however, to illustrate the principles involved.

The amounts of the different products of the lysin (Table VI) are calculated in exactly the same manner as one would calculate the analogous products of

hemoglobin from data obtained by the same methods as those used in the above lysin measurements. The analogy is made clear by the following relationships.

Lysins:

1(*a*) "Total Ly" = ($Ly_r + Ly_o + Ly_x$)
where Ly_r = reduced or active lysin; Ly_o = the reversible oxidation product of Ly_r; Ly_x = the irreversibly inactivated products of the lysin.

(*b*) $Ly_o = (Ly_r + Ly_o) - Ly_r$

(*c*) Ly_x = "Total Ly" − ($Ly_r + Ly_o$)

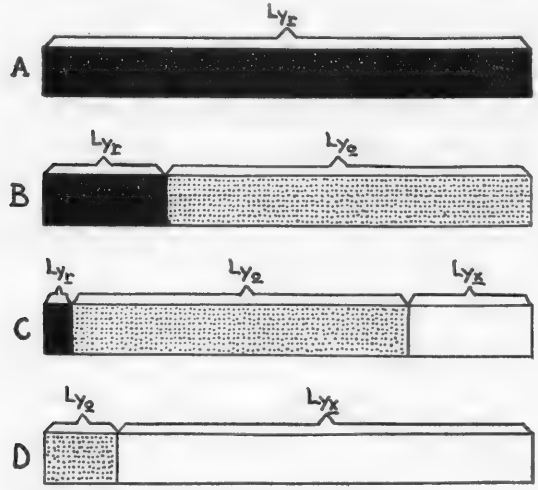

FIG. 1. Examples of the variety of mixtures of active lysin and its inactive modifications that may be present in tetanus culture fluids after exposure to different conditions. *A*, original culture fluid (not exposed to air). *B*, culture fluid aerated for 24 hours at 5°C. *C*, culture fluid aerated for 24 hours at 22°C. *D*, culture fluid aerated for 24 hours at 37°C.

Hemoglobin:

2(*a*) "Total Hb" = ($Hb_{ous} + Hb_{io} + Hb_x$)
where Hb_{ous} = "active" Hb (Hastings' (24) "ferrous Hb"); Hb_{io} = "inactive" Hb (MetHb or Hastings' (24) "ferric Hb"), the reversible oxidation product of Hb_{ous}; Hb_x = the irreversible inactive products of Hb (globin, hematin, etc.).

(*b*) $Hb_{io} = (Hb_{ous} + Hb_{io}) - Hb_{ous}$

(*c*) Hb_x = "Total Hb" − ($Hb_{ous} + Hb_{io}$)

The value of ($Ly_r + Ly_o$) is obtained by estimation of the active lysin after reduction treatment of the culture fluid, just as the value of ($Hb_{ous} + Hb_{ic}$) is obtained after reduction treatment of the hemoglobin solution.

Using the above approximate data of Table VI, it is interesting to present the graphic example shown in Fig. 1 of the variety of mixtures of active lysin and inactive lysin derivatives that may occur in deteriorated tetanus culture fluids.

Fig. 1 illustrates the fact that a series of cultures fluids with the same initial content of lysin may contain at the time of analysis, a variety of mixtures of active lysin and its inactive degradation products. The obvious conclusion, hitherto often overlooked, is that the lysin detected at any one time by the ordinary methods is determined not only by the actual lysin-producing capacity of the culture, but is equally dependent upon the conditions to which the culture fluid has been exposed previous to the time of analysis.

DISCUSSION.

Tetanolysin is inactivated when the culture fluids are exposed to air. The inactive product thus formed is the reversible oxidation product of the "active" reduced lysin since it is converted to the "active" lysin by treatment with the proper reducing agent. In addition to the reversible oxidation product, other inactive degradation products of the lysin are formed upon the aeration of tetanus culture fluids. These substances cannot be "reactivated" by reduction treatment and they probably represent degradation products of the first formed reversible oxidation product.

The methods used in the analysis of mixtures of hemoglobin and its usual degradation products are applicable to the examination of mixtures of "active" lysin and its inactive products. A complete analysis of the lysin products present in a culture fluid requires the following data: (1) the "total lysin" produced by the culture as revealed by the examination of the culture fluid before any of the lysin has deteriorated; (2) the "active" or reduced lysin present in the fluid at the time of examination; and (3) the sum of the "active" reduced lysin plus its inactive reversible oxidation product as revealed by lysin titrations of the fluid after treatment with a proper

reducing agent. If these data are at hand, it is possible to estimate the relative amounts of "active" reduced hemotoxin (Ly_r), of its inactive, reversible oxidation product (Ly_o), and of the irreversible degradation products (Ly_z) which cannot be "reactivated" by reduction treatment. It is possible that the irreversible degradation products may be formed by the deterioration of the reversible oxidation product (*i.e.*, $Ly_r \rightarrow Ly_o \rightarrow Ly_z$) in a manner analogous to the formation of globin and hematin by the splitting of methemoglobin (*i.e.*, Hb→MetHb→globin and hematin) which is the most usual course of blood pigment destruction.

SUMMARY.

A preliminary study was made of the rate of formation of tetanolysis and of the effect of glucose upon the lysin production. The heat lability of tetanolysin was next compared with that of the lysins of other bacteria. Finally the methods used to estimate the relative amounts of "active" hemoglobin and its "inactive" derivatives in deteriorated solutions of blood pigments were applied to the differentiation of the various derivatives of "active" tetanolysin present in deteriorated culture fluids.

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STUDIES ON THE OXIDATION AND REDUCTION OF IMMUNOLOGICAL SUBSTANCES.

IV. Streptolysin.

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INTRODUCTION.

The preceding papers (1–3) of this series include reports on the oxidation-reduction of the "hemotoxins" of pneumococci and of Welch and tetanus bacilli. The present paper reports a study of the "spontaneous deterioration" of streptolysin.

Streptolysin may be considered as a substance possessing hemolytic properties, which is produced during the active growth of certain streptococci in a suitable culture medium. This hemolytic action of streptococci has been the subject of many investigations during the past 25 years (4–31). The facts of essential importance in the establishment of the nature of the lysin can be obtained from the studies of Ruediger (24, 25), von Hellens (31), and Meader and Robinson (21).

Although the fact has been disputed in the literature, the actual hemolytic powers of the free lysin are independent of the presence of streptococcus cells. The lytic substance is extremely labile and under the usual conditions rapidly loses its hemolytic property. The change is commonly termed a "spontaneous deterioration," since the loss in activity has been considered to be independent of the conditions imposed during the storage of streptococcus culture fluids.

The literature (5, 8, 19, 22, 16, 24, 25) furnishes little convincing evidence that streptolysin is an antigen. However, the similarity between the "spontaneous deterioration" of the streptococcus lysin and the oxidation of the antigenic lysins of Pneumococcus and tetanus justifies the inclusion of the present paper in this series of studies on the oxidation and reduction of immunological substances.

EXPERIMENTAL.

Methods.

Strains of Hemolytic Streptococci.—Two strains of hemolytic streptococci were used in most of the experiments. Strain M was isolated by Dr. William Moss from the tissues of the neck of a child suffering from cellulitis. Strain D is a "scarlet fever strain" which has been used in the preparation of "Dick toxin." Both of these strains produce wide zones of hemolysis in less than 24 hours when grown on meat infusion blood agar plates. Other strains from the collection of the Hospital of The Rockefeller Institute were included in a number of experiments.

Hemolysis Tests.—In the usual test for hemolytic properties of streptococci in liquid systems, the whole culture is added to a suspension of red blood cells. In the present investigation, however, it was necessary to make the actual hemolysis tests with bacteria-free fluids, in order to avoid the possibility of bacterial growth and actual elaboration of lysin during the hemolysis test itself.

In view of the marked instability of the lysin, the manipulation of the culture fluid preceding the measurements of the lysin activity was kept constant throughout all of the experiments. The cultures were grown in broth under vaseline seal. Representative samples were removed and put into narrow tubes, sealed with vaseline, then centrifuged for 45 minutes at high speed. The supernatant fluids used in the tests were added to the blood cell suspensions immediately after the centrifuging process.

The hemolysis tests were made by the same methods as in the preceding studies, and consisted in "titrations" of the minimum amount of the test fluid which suffices for hemolysis of a constant volume of red blood cells. Although there are theoretical objections to this method of measurement of lysin content, the data obtained seem sufficiently accurate for the purposes of the present investigation. In a number of protocols, the results of the lysin "titrations" are recorded in terms of lysin "units." In these instances, the lysin "unit" represents the amount of lysin required to cause 80 per cent complete hemolysis of a constant volume of red blood cells. It is understood that these "units" are not presented as absolute values.

The symbols introduced in the preceding paper (3) are used to distinguish between the different modifications of the lysin; *i.e.*, Ly_r = the "active" lysin present in fluids before treatment with the reducing agent; Ly_o = the oxidized lysin which, although itself "inactive," can be converted to the originally "active" substance by reduction treatment; Ly_x = irreversibly inactivated lysin.

The "Spontaneous Deterioration" of Streptolysin and "Reactivation" of the Inactive Lysin by Treatment with Reducing Agents.

Tests were made of the possibility of "reactivating" deteriorated streptolysin by the reduction treatment employed in the preceding

investigations. The results of a typical experiment are presented in Table I.

As shown in Table I, "spontaneously deteriorated" streptolysin is "reactivated" by the reduction treatment successfully employed in the "reactivation" of the hemolytically inactive oxidation products of the previously studied bacterial lysins. It seems, therefore, that these inactive products (as in the case of the pneumococcus, Welch, and tetanus lysins) represent reversible oxidation products which can be converted by reduction, to the original, hemolytically active lysin. Since inactivation can be brought about by aeration and the

TABLE I.

"Reactivation" of "Spontaneously Deteriorated" Streptolysin by Treatment with Reducing Agents.

Amount of fluid.	Hemolytic activity of "spontaneously deteriorated" fluids of streptococcus cultures.	
	Not treated with hydrosulfite.	Treated with hydrosulfite.
cc.		
1.0	−	++++
0.6	−	++++
0.4	−	++++
0.2	−	++++
0.1	−	++++
0.05	−	++

inactive products in turn can be "reactivated" by reduction, it appears that the long discussed "spontaneous deterioration" of streptolysin is simply a reversible oxidation-reduction process.

The Influence of Exposure to Air upon the "Deterioration" of Streptolysin.

In the experiments described below, a comparison is made of the relative rates of "deterioration" of streptolysin when the culture fluid is stored in sealed tubes and in shallow layers freely exposed to air.

Cultures of two strains of hemolytic streptococci were grown in infusion broth in tubes sealed with vaseline. After 12 hours incubation, uniform portions of the culture were removed; one series of each strain was placed in narrow tubes

and sealed with vaseline; a second series was placed in Erlenmeyer flasks which exposed a large surface of the fluid to the air.

Both series were then placed in the incubator at 38°C., the usual lysin "titrations" were made at the end of 6, 12, and 24 hours.

The results of one series of tests with Strain M are given in Table II.

In Table II, the results presented in the first three columns show that while the lysin gradually "deteriorates" to inactive products when protected from air by a heavy vaseline seal, free exposure to air causes a much more rapid inactivation.

The last three columns of Table II, which record the apparent lysin content of the fluid after treatment with the reducing agent, represent

TABLE II.

Influence of Exposure to Air upon the "Spontaneous Deterioration" of Streptolysin.

	Ly_r (Lysin activity before treatment with hydrosulfite).			$Ly_r + Ly_o$ (Lysin activity after treatment with hydrosulfite).		
Amount of culture fluids.	Original culture fluid (12 hr. culture).	Fluid exposed to air 6 hrs. at 38°C.	Fluid stored in sealed tube 6 hrs. at 38°C.	Original culture fluid.	Fluid exposed to air 6 hrs. at 38°C.	Fluid stored in sealed tube 6 hrs. at 38°C.
cc.						
1.0	++++	++	++++	++++	++++	++++
0.8	++++	±	++++	++++	++++	++++
0.6	++++	−	++++	++++	++++	++++
0.4	++++	−	++++	++++	++++	++++
0.2	+++	−	++	++++	++++	++++
0.1	+	−	−	++	++	++
0.05	−	−	−	−	−	−

the sum of the active lysin (Ly_r) plus its inactive, but reversible oxidation product (Ly_o). It is important to note that the inactive products formed from streptolysin either in sealed tubes or under conditions of free exposure to air can, in each instance, be converted to the original, active lysin by reduction treatment. Since the inactive products formed are apparently the same, it is fair to conclude that the inactivation processes consist of the same type of reaction—*i.e.*, oxidation processes are responsible for the "spontaneous deterioration" of streptolysin in sealed tubes as well as in aerated fluids. Whether the oxidizing agents involved in the actual oxidations of the lysin are

the same in each case, is, of course, not proved. The essential difference, however, in the "deterioration" of streptolysin under the two sets of conditions, seems to be a difference in the rate of the reaction.

A third point of interest revealed in Table II is that in spite of the greater "deterioration" of the lysin in the aerated series, treatment with the reducing agent restores the same original lysin activity to both the sealed and aerated fluids. Thus, while in the aerated fluids much more of the active lysin has been converted to its inactive, reversible oxidation product than in the fluids held in the sealed tubes, the sum of the active lysin plus its reversible oxidation product remains the same in each instance. Apparently, under the conditions of this experiment, none of the lysin was destroyed to non-reversible, inactive products.

The results of the tests with Strain D are not recorded in Table II. The difference in stability of streptolysin in cultures of the two strains (D and M) is worthy of note, as it was constantly evident throughout the entire investigation. For example, in the above experiment cultures of Strain M lost more of the original lysin activity after 6 hours storage at 38°C., than did Strain D after 20 hours. However, the same relation between the rates of inactivation of lysin in the fluids protected from and exposed to air were evident with the latter strain as that recorded above for the more labile cultures of Strain M.

Comparison of Lysin Stability and Methylene Blue Reduction in Culture Fluids Exposed to Different Conditions.

The object of the following experiment was to compare the stability of the active (or reduced) streptolysin in different systems with the methylene blue-reducing power of the same systems.

Samples of a 12 hour broth culture of Strain M were placed under the following conditions: (1) freely exposed to the air in a shallow layer in an Erlenmeyer flask; (2) a small surface exposed to the air (the height of the cylinder of fluid was 150 mm. while the surface exposed to air was only 15 mm. in diameter); (3) sealed from air by a heavy layer of vaseline; here, too, the surface of the broth was only 15 mm. in diameter while the depth was 150 mm. Samples (4) and (5) were, respectively, the same as (2) and (3), with the exception that a suspension of *B. coli* was added to each, to furnish additional reducing action.

Sample (6) consisted of a sealed tube of the sterile culture fluid. A second series exactly the same as that described above was prepared with the exception that methylene blue was added to each of the tubes.

Series I and Series II were now placed at 38°C. At the end of 6, 12, 24, 48, and 72 hours, lysin titrations were made on Series I, and observations of the degree of reduction of methylene blue were made on Series II.

In all experiments of the type described above the lysin proved most stable in the fluids in which the reduction of the dye was most complete and most persistent. Thus, as indeed might be expected, conditions least favorable for the conversion of the colorless dye to its colored oxidation product likewise proved to be least favorable for the conversion of streptolysin to its hemolytically inactive oxidation product.

In the unsealed tubes, the reduction of methylene blue was transient; upon the cessation of growth activity of the bacteria, the dye was more or less rapidly converted to its colored oxidation product. In these tubes, in which both dye and lysin were finally oxidized, the time at which the maximum amount of lysin was in the active or reduced state, coincided with the time at which the maximum amount of the dye was in the colorless or reduced condition. Methylene blue, however, is much more readily maintained in the reduced condition than is the active streptolysin.

In the fluids which were sealed from air, the greater tendency of the lysin toward oxidation limited the application of the comparison of the persistence of methylene blue reduction with the persistence of active lysin. Even the sterile culture fluid containing no bacteria possessed sufficient reducing power to maintain the dye in the reduced state, if protected from air by a vaseline seal. Thus, it is evident that streptolysin is a substance much more readily oxidized than is methylene white, since the conversion of the colorless dye to its oxidation product was prevented in systems in which the active (or reduced) lysin was more or less rapidly converted to the inactive oxidation product.

Heat Lability of Streptolysin.

That streptolysin is a heat-labile substance has been well established in the literature (15, 14, 31, 27, 18, 20, 5, 31), but the relative degree of heat lability varied in the results of different investigators. Experiments on the heat lability

of the streptolysin in the fluids used in the present studies were made to furnish data required in the next experiment. The fluids were heated in sealed tubes in the absence of air; the reaction of the fluids was approximately pH 6.8. The results of these experiments showed that the lysin, when heated under these conditions, was completely inactivated by 5 minutes exposure to 55°C., while over half of the lysin was destroyed by 10 minutes exposure to 50°C. Thus, as shown by comparison with the results in the preceding studies (1–3) streptolysin possesses about the same degree of heat lability as the lysins of the Welch bacillus and the El Tor cholera vibrio; it is somewhat less heat-labile than pneumococcus hemotoxin, and somewhat more thermolabile than the lysin of the tetanus bacillus.

Attempt to "Reactivate" Heat-Inactivated Streptolysin by Treatment with Reducing Agents.

The heated fluids used in the experiments were exposed to 55°C. for 5 minutes (the minimum heating treatment found in the preceding experiment to effect the complete inactivation of the lysin). The "oxidized" or "deteriorated" lysin consisted of the centrifuged supernatant of a 5 day culture of the same strain. In both cases, the cultures had been grown and stored in vaseline-sealed tubes. The heating tests were likewise made in vaseline-sealed tubes.

The results of a number of experiments proved that heat-inactivated streptolysin cannot be "reactivated" by treatment with the reducing agent, sodium hydrosulfite. Thus, the heat inactivation of streptolysin is due to the formation of inactive products distinctly different from the reversible oxidation products formed during the "spontaneous deterioration" of the lysin.

Relative Amounts of Active and "Deteriorated" Lysin in Different Stages of Growth of Streptococcus Cultures.

Previous studies of streptolysin have furnished measurements not of actual lysin production, but of its relative stability at the time of the tests. In the experiment described below, streptolysin production and "deterioration" during different periods of growth of the culture were followed in a number of experiments by means of methods whereby the inactive reversible oxidation product of the lysin is detected.

Tubes containing 100 cc. of infusion broth sealed with vaseline were inoculated with 0.4 cc. of a 6 hour culture of Strain M. "Titrations" of the lysin

were made at the end of 6, 12, 24, and 48 hours. A constant volume of 4.0 cc. was maintained in the hemolysis systems; and the following increments of diluted culture fluid were tested: 0.80, 0.60, 0.50, 0.40, 0.35, 0.30, 0.250, 0.225, 0.175, 0.150, 0.125, and 0.100 cc. The hemolysis tests were incubated 45 minutes and then centrifuged.

The results of this experiment are presented in Fig. 1. Each "unit" of lysin represents the amount of lysin which causes approximately 80 per cent hemolysis of 4 cc. of a 2 per cent suspension of sheep cells. These "units" are not presented

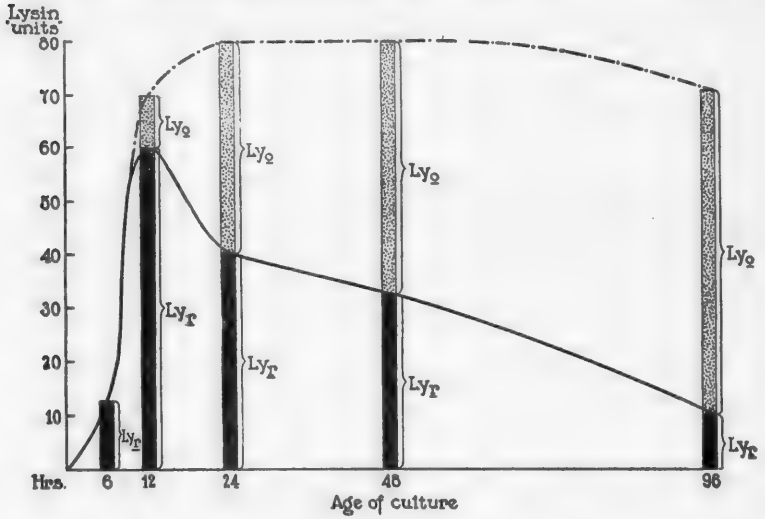

FIG. 1. Relative amounts of active and deteriorated lysin in streptococcus cultures at different ages of the culture.

The black unbroken line represents the active, reduced lysin (Ly_r); the broken line represents the sum of the active lysin plus its hemolytically inactive, but reversible, oxidation product ($Ly_r + Ly_o$). The black shaded areas represent the amount of active lysin (Ly_r) in the fluid at the time of the tests; the stippled areas represent the amount of the reversible oxidation product (Ly_o).

as absolute values, but are used merely as a convenient index for the approximate comparison of the relative lysin content of the fluid at different stages of growth of the culture.

In Fig. 1, the continuous line and the shaded areas represent the amount of lysin in the active state at the time of the test, the results having the character of those which have been reported for years in

the literature. The broken lines and stippled areas, however, represent the hemolytically inactive oxidation product of the original lysin and contribute data impossible to obtain by measurements of active lysin alone. Hence, the broken line in Fig. 1 presents a picture which more closely approaches the actual progress of streptolysin production than do any of the previous "curves of lysin production."

While there is little or no "deteriorated" lysin present during the period of maximum growth of the streptococci, the "deterioration" has begun, in this particular culture, by the time of the 12 hour test. Moreover, it is evident in Fig. 1 that the amount of active lysin may begin to diminish before the maximum amount of "total lysin" ($Ly_r + Ly_o$) has been liberated into the medium. This is an important fact and demonstrates that, at least with certain strains of streptococci, the measurement of lysin by the usual method never furnishes the value of the total amount of lysin actually elaborated by the culture. This has been especially true with our Strain M, with which it has never been possible to obtain samples of culture fluid in which there was no "deteriorated" lysin—*i.e.*, fluids in which Ly_r was not less than ($Ly_r + Ly_o$). We have conducted a number of experiments of the type described above with different strains of streptococci. The fact that with some strains the lysin "deterioration" begins before the liberation of the maximum amount of "total lysin" while with other strains the lysin proves more stable, is evidence that the usual measurements of active lysin cannot furnish a proper basis of comparison of actual lysin production by different streptococci.[1]

[1] If practically all of the lysin had "deteriorated" in the fluid tested, the usual method of lysin measurement might lead one to believe that the culture tested was totally devoid of lysin-producing powers, or at least that no lysin had been produced in the tested culture medium.

It would be a mistake however, to emphasize this possibility as a likely source of error in determining whether or not a particular strain of streptococcus is a "hemolytic streptococcus." As a matter of practice, the criterion used in the separation of "hemolytic" streptococci from "non-hemolytic" strains is the production of distinct wide zones of hemolysis during the early growth of the colonies on a blood agar plate (the "beta hemolysis" of Smith and Brown). Where hemolysis in liquid systems is used as a descriptive or systematic criterion

In Fig. 1, it is also significant that while the amount of active lysin rapidly diminishes, the sum of the active lysin and its inactive, reversible "deterioration" product ($Ly_r + Ly_o$) remains constant for several days. In most of our experiments, the sum of these two products remained the same for 5 or 6 days. The fact that still older culture fluids were found not to regain all of their original active lysin content after the reduction treatment can be explained by the formation of irreversible degradation products (Ly_x) which cannot be converted by reduction to the originally active lysin.

The Influence of Yeast Extract in the Culture Medium upon the Production and Stability of Streptolysin.

As suggested by Meader and Robinson's (21) work, one of the constituent which is required for streptolysin production bears certain resemblances to the so called "accessory substances." The work of Avery and his associates (32), as well as that of other investigators, has demonstrated the marked effect which yeast extract may have upon microbial activity. Experiments on the influence of yeast extract upon the formation and stability of streptolysin seemed especially pertinent to the present investigation, since certain constituents of yeast extract had been found in previous studies (33) to have an intimate relation to the oxidation-reduction activities of different bacteria.

In experiments of the type described below, comparisons were made of the lysin produced in cultures grown in "unfiltered" muscle infusion broth with that produced in the same broth enriched by the addition of yeast extract. In the comparison of lysin production in different culture media, the methods developed in the preceding papers offer certain definite and obvious advantages since they furnish values more closely approaching the "total lysin" actually produced in the tested medium.

"Unfiltered" Muscle Infusion Broth.—Finely minced, fat-free, heart muscle was infused in slightly acidified tap water for several hours and then gradually

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the whole culture is added to a suspension of blood cells, in place of the bacteria-free fluids used in the hemolysis tests in this study. Under these conditions, with a reasonably young, whole culture the danger of failure to detect lytic capacity is much more remote than would appear from the results of the preceding experiments, in which all tests were made with the bacteria-free culture fluids.

brought to a boil. Peptone and salt were added to the decanted infusion, and the broth, still slightly acid, was autoclaved for 10 minutes. The broth was adjusted to pH 7.8, let stand for several hours, and the clear supernatant siphoned off. This medium was finally sterilized in the autoclave for 8 minutes in tubes containing 100 cc. of the medium. After sterilization, a layer of sterile vaseline was added to the hot broth.

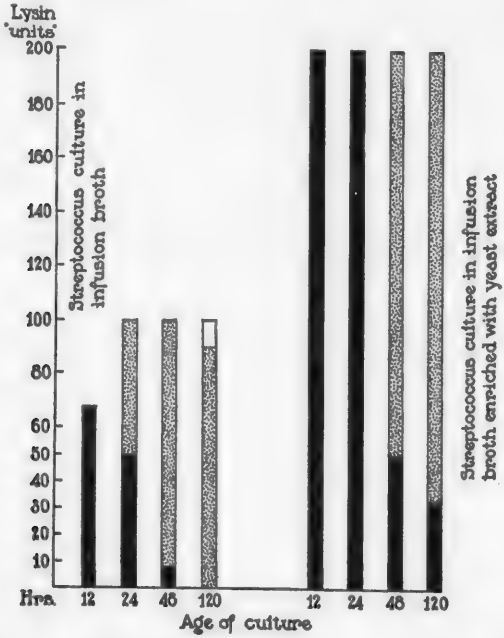

FIG. 2. The influence of yeast extract in the culture medium upon the production and stability of streptolysin.

The black shaded areas represent active (or reduced) lysin (Ly_r). The stippled areas represent the hemolytically inactive, reversible oxidation product (Ly_o) of the original or reduced lysin. The white area represents hemolytically inactive degradation products (Ly_x) of the lysin which cannot be "reactivated" by reduction treatment.

Broth Enriched with Yeast Extract.—Yeast extract broth was prepared by adding 7.5 cc. of yeast extract to each of several tubes of the above described meat infusion broth. The yeast extract was furnished by Dr. O. T. Avery of the Hospital of The Rockefeller Institute. The method of preparation of the yeast extract has been described in previous papers (34).

The lysin measurements were made by the methods used in the preceding experiments. Human blood cells were used in these experiments.

The results of an experiment performed as described above are presented in graphic form in Fig. 2. The same lysin "units" are employed as those used in the presentation of the experiment illustrated in Fig. 1.

The results of these experiments (Fig. 2) show that the addition of yeast extract to the medium enhances lysin formation, since it causes an increase in both the rate of lysin elaboration and the "total lysin" production. However, it is more pertinent to the present study to observe that the lysin after its elaboration "deteriorates" much more slowly in the enriched medium. For example, although much of the lysin formed in the unenriched culture medium "deteriorated" between the 12 and 24 hour tests, during the same period in the yeast extract broth, none of the lysin was converted to inactive oxidation products.

While certain constituents of yeast extract may have a specific effect upon the formation of streptolysin, the differences observed between the media used in the above experiment could also be explained by known effects of yeast extract upon bacterial growth activity. The increased rate of lysin formation as well as the greater total production of lysin may be due to a more rapid rate of growth of the streptococci in the enriched medium. It is probable that the deterioration of the lysin in any medium depends upon the loss in the reducing activity which was maintained during the period of active bacterial growth. If the yeast extract enables the streptococci to retain their life or growth activity for a longer period, the increased stability of the lysin is easily explained by the more persistent reducing conditions maintained in the yeast broth.

The difference in stability of the lysin in the two culture media offers another example of the advantages of the method of lysin measurement described. In the case of the unenriched broth, with certain strains of streptococci, a significant amount of active lysin is always converted to inactive products before the total amount of lysin actually elaborated has been liberated into the medium. Since in the yeast extract cultures the lysin proved more stable, it is obvious that a fair comparison of the actual production of lysin in the two media must take into account the inactive products formed by the deterioration of originally active lysin.

The Inhibition or Prevention of the Usual "Spontaneous Deterioration" of Streptolysin by Storing the Culture Fluids in a Sealed System Containing Sodium Hydrosulfite.

From analogous studies on hemoglobin (35), it seemed possible that the "spontaneous deterioration" of the bacterial lysin might be prevented if reducing conditions were imposed during its storage. To test the validity of this assumption, sodium hydrosulfite was added to streptococcus culture fluids in sealed tubes; and the stability of the lysin under these conditions was compared with the stability of the lysin in the same culture fluids to which no reducing agents had been added.

Cultures of Strain M grown in unenriched muscle infusion broth were chosen, as the lysin of this strain had proved most labile throughout all of the preceding experiments.

Portions of the streptococcus culture fluid, taken after stirring the culture, were assumed to be representative samples containing equal amounts of the lysin. The following series of tubes were then prepared:

1. Culture Fluid Alone.—4 to 5 cc. of the culture fluid were placed in narrow agglutination tubes so that the tubes were filled to within $\frac{1}{2}$ to $\frac{3}{4}$ of an inch from the top.

2. Culture Fluid Plus Hydrosulfite.—Tubes containing 25 mg. of $Na_2S_2O_4$ had previously been sterilized in hot air at 140°C. To these tubes, measured 5 cc. portions of the culture were added, which gave a concentration of 0.5 per cent of hydrosulfite.

pH of the Fluids.—The reaction of the culture fluid alone was approximately pH 6.6. The addition of the hydrosulfite resulted in a drop in pH, so that the culture plus hydrosulfite was about pH 5.9.

In an attempt to obtain a series containing the reducing agent but at the pH of the culture fluid alone, sterile NaOH was added to six of the hydrosulfite series. Unfortunately, through an error, too much alkali was added, and the final reaction at which these tubes of fluid were stored was about pH 9.0.

Sealing of the Tubes.—The tubes were sealed with care to make the exclusion of air as absolute as possible. The narrow tubes were completely filled with melted sterile vaseline immediately after the addition of the culture fluid. The excess vaseline escaped through the groove when a sterile, grooved cork stopper was carefully forced into the tube. The cork was finally completely sealed with sealing wax.

Storage of the Fluids.—A series of tubes containing culture fluid alone and tubes containing culture fluid plus $Na_2S_2O_4$ were stored at 38°C.; a second series were stored at room temperature. The temperature of the room varied con-

siderably; during the first 5 days it was approximately 25°C.; between the 5th and 21st days, it reached 32°C. on several occasions. The 140 day sample unfortunately was exposed to several prolonged periods of hot weather in which the temperature was frequently 35°C.

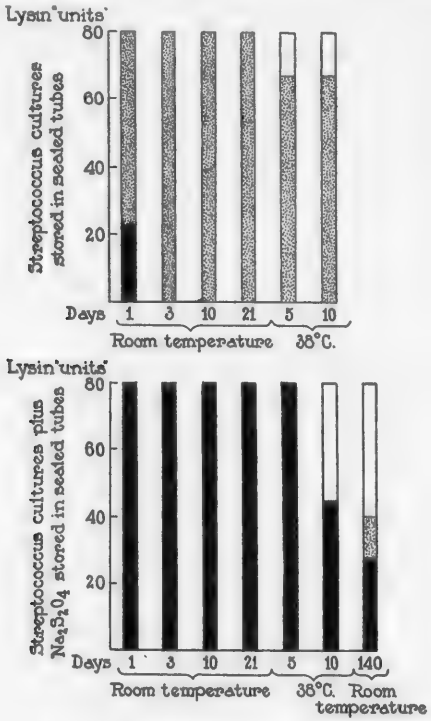

FIG. 3. Inhibition of the "spontaneous deterioration" of streptolysin by storing the culture fluids in a sealed system containing sodium hydrosulfite.

The black areas represent the reduced or active lysin (Ly_r); the dotted or stippled areas represent the hemolytically inactive reversible oxidation product of the lysin (Ly_o); the white areas represent further, inactive degradation products (Ly_a) which cannot be "reactivated" by reduction treatment.

Lysin Measurements.—The tubes of culture fluid were centrifuged and the bacteria-free supernatants used in the tests. 2 cc. of the test fluid were placed in each of two tubes; one of the tubes already contained 2.0 cc. of 0.1 M phosphate solution; 2.0 cc. of 1.0 per cent $Na_2S_2O_4$ in 0.1 M phosphate solution were

added to the other tube. After allowing time for reduction of the second tube, aliquots of these mixtures were used in the lysin titrations. Tests of the first of these mixtures represent measurements of the active lysin alone; the tests of the second of the above mixtures include not only the active lysin but the inactive, reversible oxidation product.

Controls in which uninoculated broth was stored in sealed tubes containing $Na_2S_2O_4$ were included to eliminate the possible formation of hemolytic substances by action of hydrosulfite upon the culture medium itself.

The results of this experiment are presented graphically in Fig. 3. In this figure, the measurements of lysin content of the fluids are recorded in terms of "lysin units," one "unit" representing the amount of lysin required to cause approximately 80 per cent hemolysis of 3 cc. of a 2 per cent suspension of blood cells.

In streptococcus cultures stored in sealed tubes under the usual conditions (*i.e.*, containing no added reducing agent), the active lysin is always soon converted to inactive products. In the experiment presented (Fig. 3) the fluid had become entirely devoid of lytic action after 3 days storage at room temperature. In fact, the "spontaneous deterioration," as might be expected from previous results (Fig. 1) with this especially labile strain, had already begun at the beginning of the storage experiment.

In contrast to the usual rapid "deterioration" in streptococcus cultures, the series in which hydrosulfite had been present during storage retained their entire original lysin activity for at least 21 days days storage at room temperature, and for over 5 days at 38°C. Upon addition of the hydrosulfite at the beginning of the storage period, the hemolytically inactive oxidation product already formed in the fluid was reduced back to the original, active lysin. Then after the system was sealed the excess reducing agent maintained conditions which made impossible the usual "spontaneous" deterioration of lysin during the subsequent storage. It is evident that under these conditions (*i.e.*, stored in a sealed system in the presence of an active reducing agent), streptolysin, a classically labile bacterial product, would appear to be a relatively stable substance.

The results obtained with the hydrosulfite fluids after storage for 10 days at 38°C. and 140 days at "room temperature" introduce certain complications which, however, do not detract from the important relation evident in the results already discussed. Although some active lysin still persisted in these fluids, a

considerable portion has become inactive. It is possible that the failure of these fluids to retain their original lysin activity is due to some effect of prolonged exposure to high temperatures upon the hydrosulfite, as well as upon the lysin. (During the later part of the storage of the 140 day "room temperature" tube, it was subjected for several days to a temperature of 35°C.) It is significant that the lysin in these instances was converted to irreversible products rather than to the usual reversible oxidation product.

DISCUSSION.

Streptolysin is an extremely labile bacterial product. Under ordinary conditions, its loss of activity is so rapid and so pronounced that the hemolytic property of streptococcus culture fluids is recognized throughout the literature as "*une propriété éphémère*" (13).

In the present paper, it is shown that the hemolytically inactive product formed by this "spontaneous deterioration" can be "reactivated" by treatment with a chemical reducing agent. If the "reactivation" represents the reduction of an inactive, reversible oxidation product to the original active substance (reduced streptolysin), the process involved in the usual "spontaneous deterioration" of streptolysin can be regarded as an oxidation reaction which yields a reversible product.

Free exposure to air is not required for the oxidation of streptolysin. Although aeration of the culture fluid in shallow layers results in a much more rapid inactivation of the lysin, culture fluids held in tubes sealed from air gradually lose their hemolytic activity. The "deterioration" of streptolysin in tubes protected from air is in marked contrast to the stability of the lysins of Welch and tetanus bacilli, which, at least in culture fluids more acid than pH 7.0, remain active for months if the culture tubes are sealed with vaseline. The inactivation of the lysin in sealed tubes is due to the formation of a reversible oxidation product and hence, must be accepted as an oxidation process which yields a product identical or similar to that formed in aerated culture fluids. It is quite possible that the exclusion of air was not absolute in any of our experiments, and in the absence of exact controls of the gas content of the culture fluids, one cannot meet the question of whether or not traces at least of molecular oxygen are required for the oxidation of streptolysin.

The usual method for measuring bacterial lysins in culture fluids detect only the lysin which is in the active state at the time of the test. The methods previously employed in the studies (2, 3) of the Welch and tetanus lysins furnish in addition to measurements of active lysin, measurements of total lysin production and of the ratio of active to total lysin.

Applications of these methods have furnished a picture more complete than those existing heretofore of the actual course of lysin production and of lysin "deterioration" at various stages of growth of different strains of streptococci. The most important facts revealed were the following: (1) the content of active lysin (Ly_r) frequently decreases before the maximum amount of total lysin ($Ly_r + Ly_o$) is liberated into the medium; (2) the ratio $\left(\frac{Ly_r}{Ly_r + Ly_o}\right)$ varies with different strains and with different conditions to which the culture is subjected. These two facts in themselves are sufficient evidence that methods detecting only active lysin (Ly_r) cannot furnish a proper basis for comparisons of actual lysin production, either in experiments with different strains under the same conditions or in experiments with the same strain under different conditions.

The "spontaneous deterioration" of streptolysin, like the usual "spontaneous deterioration" of hemoglobin, consists in the conversion of an active substance to its inactive reversible oxidation product. As previously shown for hemoglobin (35), the formation of the inactive oxidation product is inhibited or wholly prevented in sealed systems containing an active reducing agent. For example, with one of the more labile strains studied, all of the active lysin "deteriorates" to inactive products in 2 or 3 days when stored at room temperature in sealed tubes containing no added reducing agent; on the other hand, in sealed tubes to which hydrolsulfite had been added, the same culture fluids were stored for more than 21 days without loss in the apparent content of active lysin and without formation of a detectable trace of the inactive "deterioration" product. Apparently, therefore, if reducing conditions of sufficient intensity be maintained in the culture fluid, the ratio of active lysin to total lysin $\left(i.e., \frac{Ly_r}{Ly_r + Ly_o}\right)$ remains constant for a considerable

time at a value approximating unity. It is probable that the reducing conditions established by the streptococcus cells during the period of maximum growth diminish in intensity upon the cessation of the bacterial growth activity and this failure of the bacterial culture to maintain the strong reducing conditions which obtained during the period of active lysin elaboration is the cause of the conversion of active lysin to its inactive oxidation product (*i.e.*, a decrease in the ratio $\left(\frac{Ly_r}{Ly_r + Ly_o}\right)$. From this point of view, one would expect the conversion of the active lysin to its oxidation product to be inhibited in any system in which reducing conditions are maintained for a longer period. If the ratio of active lysin to total lysin $\left(\frac{Ly_r}{Ly_r + Ly_o}\right)$ be accepted as an index of reducing conditions in the culture fluid, it is interesting to observe that observations of an unusual persistence of active streptolysin in certain cultures (as for example, the yeast extract cultures of our Strain D) indicate that reducing conditions of sufficient intensity are maintained for a longer period in these cultures.

It has been mentioned before that the lysin of streptococcus "deteriorates" in vaseline-sealed culture fluids, while the lysin in culture fluids of pneumococci (36), tetanus (3), and Welch bacilli (2) are relatively stable if similarly protected from air. An explanation of the greater tendency of streptolysin toward "deterioration" involves two factors: either reducing conditions of sufficient intensity are maintained for a longer period in the cultures of the other bacteria, or streptolysin itself is a substance intrinsically more easily oxidized than the other lysins. The importance of the first factor has already been illustrated by the relative stability of the usually labile streptolysin when stored in a sealed system containing an added chemical reducing agent. A true evaluation of the second factor must depend upon measurements of the reducing intensities obtaining in the respective culture fluids. However, the fact that streptolysin is converted to its oxidation product in systems of sufficient reducing power to prevent a similar conversion of methylene white indicates that the lysin itself is a relatively easily oxidized substance.

SUMMARY.

The "spontaneous deterioration" of streptolysin has been studied by the methods already used in investigations of the oxidation-reduction of blood pigments and of the lysins of other bacteria. From the results of this study, it may be concluded that the commonly observed "spontaneous deterioration" of streptolysin consists in the conversion of the originally active lysin to a hemolytically inactive oxidation product. This process is a reversible one, and the activity of the lysin is restored by reduction.

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THE FUNDAMENTAL PROPERTIES OF THE FIBROBLAST AND THE MACROPHAGE.

I. The Fibroblast.

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Plates 6 and 7.

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Tissue cells, like bacteria, must be defined not only by their morphology but also by their physiological properties. A complete knowledge of the biological characteristics of the various cell types would render possible the prediction of the behavior of the cells themselves, as well as their identification. We are still ignorant of these essential properties of the fibroblast and the macrophage, although their anatomy and their staining reactions have been extensively studied during the last 20 years. In his fundamental work on connective tissue, Renaut discovered the characteristic manner in which both types of cells respond to neutral red.[1] His findings were confirmed by Evans and Scott, who ascertained the differential reaction of the fibroblast and the macrophage, not only to neutral red, but also to a number of acid dyes.[2] The protoplasmic structures of these cells have also been minutely described by Maximow,[3] and by Lewis and Lewis[4,5] in their painstaking studies of mitochondria and neutral red vacuoles in tissues surviving *in vitro*. As these experimenters did not extend their investigations beyond the morphological realm on account of the inadequacy of their techniques, the basal characteristics of the fibroblast have remained unknown. However, a study of these

[1] Renaut, J., *Arch. anat. micr.*, 1906–07, ix, 495.

[2] Evans, H. M., and Scott, K., *Carnegie Institution of Washington, Pub. No. 273, Contributions to Embryology*, 1921, x, 3.

[3] Maximow, A., *Beitr. path. Anat. u. allg. Path.*, 1902, suppl. 5, 1.

[4] Lewis, M. R., and Lewis, W. H., *Am. J. Anat.*, 1914–15, xvii, 339.

[5] Lewis, W. H., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 81.

properties became possible as soon as more elaborate procedures[6] were developed; namely, the isolation of cells in pure cultures, the use of both nutrient and preservative media, the maintenance of a constant composition of the latter, the measurement of the rate of growth, the cinematographic recording of cell locomotion and of the movements of the protoplasmic constituents, etc. In the experiments which are the subject of the present article, an attempt has been made to ascertain the fundamental properties of the fibroblast and to correlate both its functional state and its changing appearance.

Characteristics of the Colonies of Fibroblasts.

Fibroblasts never grow as isolated units, but form a dense tissue. When a colony is embedded in plasma, the fibroblasts migrate into the new medium without losing contact with the tissue from which they come. The area covered by the colony gradually increases and the cells never scatter through the medium, as macrophages do. On the advancing edge of the tissue, a few isolated cells may be observed, but they are generally united by their processes to neighboring cells. A colony is circular, and the long axis of the cells of its advancing edge is generally parallel to its radius. The fibroblasts remain in intimate reciprocal contact on all sides and multiply actively when packed together. This characteristic probably belongs to all fixed connective tissue cells, as Rous has found that isolated mesenchyme cells in suspension in a medium unite again to form a reticulum.[7] The aptitude to form a tissue establishes a fundamental difference between fibroblasts and macrophages since the macrophages live as independent units and die if they congregate in dense masses. When two colonies of fibroblasts grow in a single flask at a short distance from one another, they display a tendency to unite their edges. It seems as if the cells of one colony attract the cells of the other, and the rate of growth of the parts of the tissue that are at close range slightly increases until the union of both colonies takes place.

Fibroblasts in pure culture never invade the entire medium. When a small colony is implanted in the center of a flask 50 mm. in diameter,

[6] Carrel, A., *Physiol. Rev.*, 1924, iv, 1; *Brit. Med. J.*, 1924, ii, 140.

[7] Rous, P., and Jones, F. S., *J. Exp. Med.*, 1916, xxiii, 549.

and is left undisturbed in the coagulum, while the fluid medium consisting chiefly of embryonic juice is changed every 2 days, it grows at the optimum rate, and the curve expressing the increase in area is a parabola. After about 2 weeks, when the diameter of the colony has reached approximately 15 mm., the growth stops spontaneously. The tissue will resume its activity only after division and transplantation into another flask. While fibroblasts never cover a large area of the coagulum, macrophages often invade the entire medium in less than 3 weeks. The impossibility of a colony of fibroblasts growing beyond a certain size may be attributed to its peculiar architecture and to the solidarity of the cells. When two cells are united by their processes, and one is punctured with a needle and killed, the death of the other cell follows after a few seconds, as Chambers has shown.[8] Although Lewis believes that there is no anatomical communication between cells united by their processes, it is obvious that they are in close relation.[9] This condition is probably required for cell proliferation. Fischer found that cells isolated in a culture medium do not proliferate.[10] In order for multiplication to occur, fibroblasts must be in close contact. This inherent property of forming a tissue may cause the limitation in size of the colonies. After the colony has reached a certain thickness, its central part probably becomes isolated from the food supply and degenerates, and toxic substances are set free which stop cell proliferation. We have often observed that the formation of a thick center in a colony is accompanied by marked changes in the appearance of the peripheral cells, which display a tendency to grow in scattered formation. Twice in the course of 14 years, a transformation of these scattered cells into macrophages occurred.

Fibroblasts have a special affinity for other fibroblasts, but not for epithelial cells. When a colony of pure epithelium is grown side by side with a colony of fibroblasts, the fibroblasts rapidly surround the epithelium, as was shown in an experiment by Ebeling and Fischer.[11]

[8] Chambers, R., in Cowdry, E. V., General cytology, Chicago, 1924, 242.

[9] Lewis, W. H., and Lewis, M. R., in Cowdry, E. V., General cytology, Chicago, 1924, 393.

[10] Fischer, A., Tissue culture; studies in experimental morphology and general physiology of tissue cells *in vitro*, Copenhagen, 1925, 155.

[11] Ebeling, A. H., and Fischer, A., *J. Exp. Med.*, 1922, xxxvi, 285.

The section of the culture indicated that nowhere had the fibroblasts mixed with the epithelial cells. The epithelium had grown in a gland-like formation, without being penetrated by the fibroblasts. Generally macrophages do not remain among fibroblasts. They migrate more quickly and scatter freely through the medium far from the colony of fixed cells.

Rate of Growth of the Colonies.

The rate of growth of the colonies under various conditions has been ascertained by a large number of measurements in the course of the last 14 years. In a medium composed of 1 volume of adult chicken plasma and 1 volume of juice from 8 or 10 day old chick embryos and kept at a temperature of 39°C., the colonies double in mass every 48 hours. This means that a weight of protoplasm equal to that of the original colony is synthetized from the medium constituents during this short period of time. The epithelial cells respond also to the presence of embryo juice by a great increase in their activity. But their rate of proliferation never equals that of fibroblasts. In the same cultural conditions, the macrophages cease to multiply. The fibroblast is the fastest growing cell that has so far been isolated. The velocity of cell multiplication depends at first on the age of the animal or the condition of the culture from which the colony is obtained.[12] Fibroblasts removed from an embryo or a young culture multiply *in vitro* more rapidly than those from an older embryo or older culture. But the activity of fibroblasts of different original growth energy and cultivated in identical media has a tendency to become uniform. After they have been cultivated for a few days or weeks in the same substances, they proliferate at the same speed.[13] The rate of growth of a colony at a given instant is a function of its activity at the preceding instant, and of the concentration of the growth-activating and inhibiting substances in the medium. As long as the composition of the medium remains constant, the rate of growth does not vary. After more than 14 years of life *in vitro*, a strain of fibroblasts is growing at the same speed as during the first years of cultivation.

[12] Carrel, A., *J. Exp. Med.*, 1913, xvii, 14.
[13] Carrel, A., *J. Exp. Med.*, 1923, xxxviii, 521.

While multiplying at their optimum rate, the fibroblasts store up some food material which is responsible for the growth energy they display when transplanted into a medium composed chiefly of Tyrode solution. During starvation, their residual energy permits the increase in area of the colony for a period varying from 6 to 8 days. The quantity of the reserves which the cells accumulate depends on the time they spend in a medium containing embryo juice and on the concentration of the juice in the medium. A maximum is rapidly reached beyond which no more food is stored up. The residual energy of the fibroblast appears to depend on its specific property of accumulating a certain amount of food material while being cultivated in a nutrient medium.

Mode of Locomotion of the Fibroblast and Movements of the Protoplasmic Structures.

The locomotion of the cells was studied on cinematographic films of pure cultures. The photographs were taken at a rate of three to six exposures per minute during 24 or 48 hours. When the films were projected at a rate of ten exposures per second, the velocity of the motion was increased from 100 to 200 times. As the magnification was only 70 diameters, a large area of the medium could be kept under observation. The cells progressed in straight lines. At a temperature of 39°C., their speed was uniform and reached approximately 33.3μ per hour. Practically all the cells of a colony moved away from the center. Their activity was polarized, the distal end of the cells projecting its processes into the medium, while the proximal end merely followed. The front processes flowed through the medium, adhered to some of the structures, and kept the body of the cell stretched. If one of the processes lost its hold on the fibrin of the medium, the body suddenly jerked toward the processes that were still adherent to the clot. The protoplasm streamed through the front process, then the nucleus and the cytoplasmic structures of the body glided forward, dragging the rear process. Under dark-field illumination, the body and the processes were defined by a sharp line of beautiful geometrical design. At the end of the processes, which were always open, the boundary between cytoplasm and medium appeared very faint and irregular. The cytoplasm flowed between the lateral

boundaries of the anterior process through its open end. Continuous streaming of the cytoplasm in a straight line toward the free medium is a characteristic of the fibroblast. In exceptional instances, cells were observed moving backward. They almost never turned in a circle. Practically every cell in the peripheral area of a colony showed a very active outer pole and an inactive inner pole. The pictures emphasized the profound difference between this mode of locomotion and that of the macrophages. The latter cells are encircled by a thin undulating membrane which moves unceasingly and molds its folds on the surrounding bodies.

The motion of the protoplasmic structure was studied on pictures taken with a magnification of 300 to 400 diameters and at a rate of sixty exposures per minute. When the film was projected at the rate of ten exposures per second, the velocity of the motion was increased only ten times. It became possible to ascertain the relative displacements of the nucleus and of the cytoplasmic organs on preparations stained with neutral red, and on unstained preparations under dark-field illumination. When the motion of the cells was observed with a tenfold increase, the nucleus did not seem to undergo any displacement or change in form. On the contrary, the nucleoli modified their shapes continually, as already observed by Lewis.[14] The fat globules, which were generally present in small number in the cells fed upon tissue juice, had no motion of their own. The larger they were, the more inert they appeared. The neutral red vesicles moved incessantly, and the amplitude of their oscillations was small. Nucleus, fat globules, and neutral red vesicles formed the main part of the body and followed the displacement of the cell as a whole, without their relative position being altered to any great extent. The more fat a fibroblast contained, the less agile it became. The small red granules could be divided into two classes according to their mode of locomotion, the stationary granules, and the wandering granules. Some of the stationary granules were located in the stained vesicles. Their characteristics have been minutely described by Renaut,[1] and later by Lewis.[5] Some others were apparently free within the body or the processes. The tracings made of the successive positions they occu-

[14] Lewis, W. H., *Anat. Rec.*, 1923, xxvi, 15.

pied in a number of exposures showed that they covered an oblong or circular area and that they moved inside an invisible vacuole or vesicle. The wandering granules could travel all over the cell from the central part of its body to the far end of the processes and back. Under dark-field illumination, they could not be mistaken for round mitochondria because, as already observed by Lewis,[5] the minute mitochondria are not usually found in fibroblasts that show the small neutral red granules. With dark-field illumination, the unstained filamentous mitochondria were observed to move, as already described.[4] The swift motion of the wandering granules and of the mitochondria indicated the location of free paths within the body. The wandering granules passed rapidly by the stationary granules, which moved back and forth in invisible vacuoles. They might also travel around the neutral red vesicles and the fat globules of the body. It is obvious that certain granules move freely around the bodies described by Renaut[1] and Evans[2] as normal segregation apparatus, and by Lewis[5] as degeneration vacuoles. The vacuoles cannot be considered as free spaces, full of fluid, in a jelly-like cytoplasm. They are evidently vesicles, empty or containing a granule. As the path of the wandering granules is irregular and broken, it is probable that the cytoplasm has a sponge-like structure. It is not a gel, a fact shown by the rate of motion of the filamentous mitochondria, as well as by that of the minute red granules. The body of the fibroblast appears to be composed mainly of small vesicles, that do or do not stain with neutral red, and between which fat globules may accumulate. In the perivesicular fluid wander the small neutral red granules and the filamentous mitochondria. The conception of the cytoplasm as a mass of jelly containing some vacuoles full of fluid should be discarded as inaccurate.

Food Requirements of the Fibroblast. Its Susceptibility to Arsenous Oxide.

Fibroblasts do not feed on plasma, egg albumin, egg yolk, amino acids, or broth.[15] They synthetize protoplasm exclusively from sub-

[15] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 317, 599; 1923, xxxviii, 487.

stances contained in the juice of chick, mouse, guinea pig, and rabbit embryos.[16] This characteristic distinguishes fibroblasts from macrophages which die when cultivated in pure embryo juice, and multiply in a medium composed exclusively of plasma.[17] Both fibroblasts and epithelial cells possess the property of feeding upon embryo juice. But the effect of the juice is more marked on connective tissue than on epithelium. Fibroblasts also have the property of proliferating when fed upon Witte's peptone.

Fibroblasts and macrophages differ in a striking manner regarding their susceptibility to arsenous oxide. Arsenous oxide in concentrations varying from 1/50,000 to 1/3,000,000 was added to the medium of a 14 year old strain of fibroblasts, and the rate of growth of the colonies was measured. When the arsenic was present at 1/3,000,000 in the medium, the speed of proliferation decreased slightly. The growth completely stopped when the arsenous oxide reached a concentration of 1/800,000. This concentration of arsenic was entirely innocuous to macrophages, which were not killed by a concentration of 1/200,000. The susceptibility of fibroblasts to arsenous oxide renders it possible to obtain pure cultures of macrophages from mixed cultures by using media containing 1/300,000 arsenous oxide.

Relations between Protoplasmic Structures, Nature of Medium, and Rate of Growth of Colonies.

The colonies were composed of chicken fibroblasts belonging to a 14 year old strain, or to strains obtained more or less recently from the subcutaneous connective tissues of adult animals. They were cultivated in three different media, chiefly composed of serum or Tyrode solution or embryonic tissue juice, according to two techniques that have been previously described.[18] The rate of growth of the colonies was measured by the ordinary methods.[19] When the fibroblasts had multiplied for a few days and the rate of growth had been ascertained, they were transferred onto a cover-glass for examination. From 1 to

[16] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1923, xxxviii, 499.
[17] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxvi, 365.
[18] Carrel, A., *J. Exp. Med.*, 1923, xxxviii, 407.
[19] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

48 hours after the slides were prepared, the tissues were stained with 1/20,000 Janus green and 1/20,000 neutral red, and camera lucida drawings were made of the cells at a magnification of 1600 or 3200 diameters.

1. Conditions of Neutral Red Vesicles and Mitochondria in a 14 Year Old Strain of Fibroblasts.—The strain has been kept for 14 years in equal volumes of chicken plasma and embryonic tissue juice. The rate of growth is such that the colonies double in volume every 48 hours. Tissue fragments in a condition of optimum activity were stained with neutral red and Janus green, and camera lucida drawings made.

Generally, the length of the cells varied from 100 to 140μ, and that of the nucleus from 18 to 22μ. Neutral red vesicles and small fat globules were located in unequal quantities in the cytoplasm at both ends of the nucleus. The vesicles were from 1 to 1.5μ in diameter. Their number varied approximately from 50 to 100. They often contained a small red granule. There were also minute wandering granules in the body of the cell and in its processes. Their diameter was less than 1μ. The vesicles accumulated usually in the anterior part of the body, probably around the centriole, while the mitochondria were located around the nucleus, in the processes, and between the vesicles. They were long, filamentous, and of even caliber. The dumb-bell, vesicular, and other abnormal forms described by Lewis and Lewis[4] were not present (Fig. 1).

2. Neutral Red Vesicles, and Mitochondria in Fibroblasts from Adult Connective Tissue.—Fragments of subcutaneous connective tissue were extirpated from young adult chickens. They were cultivated in a medium composed of equal parts of plasma and embryonic tissue juice. Every 2 days, the tissues were removed from the containers, washed in Tyrode solution, and transferred to a fresh medium. From 24 hours to 2 weeks after the extirpation of the tissues, the cultures were stained with neutral red and Janus green, separately or in combination, and camera lucida drawings made of the cells.

Subcutaneous Connective Tissue from an Adult Chicken, Cultivated in Equal Volumes of Plasma and Embryonic Juice.

Experiment 1.—Culture 2956-C, Jan. 14, 1926. Jan. 15, camera lucida drawings. Length of nucleus, 18μ; length of cells, 90μ; no neutral red vesicles; 2 to 5 small red granules wandering through the processes; 51 fat globules (Fig. 2).

Experiment 2.—Culture 3338-C, Feb. 24. Feb. 26, stained with neutral red and Janus green; camera lucida drawings. The cells which were close to the original fragment were 120μ in length, and the nucleus was 15μ; the cells contained 20 small neutral red vesicles about 0.5μ in diameter; 4 or 5 wandering red granules; about 15 small fat globules; 35 mitochondria averaging 2.5μ in length (Fig. 3). Some of the cells which had wandered out into the medium a short distance from the original fragment of tissue showed about 37 neutral red vesicles 0.75μ in diameter; 4 or 5 wandering stained granules; 35 mitochondria, 2.5 to 3μ in length (Fig. 4).

Experiment 3.—Culture 3387-C, Mar. 2. Mar. 4, camera lucida drawings. 20 to 25 small neutral red vesicles, 0.5μ in diameter; 6 to 8 wandering granules; 50 to 60 mitochondria, averaging 1.75μ in length; some cells show a few fat globules; length of nucleus averages 15μ; length of cell, 85 to 95μ (Figs. 5 and 6).

Experiment 4.—Culture 2876-C, Jan. 5. Jan. 8, first passage, Culture 2901-C. Jan. 9, camera lucida drawings; length of cell, 75μ; length of nucleus, 15μ; 25 vesicles, 1μ in diameter; about 10 fat globules; a few wandering neutral red granules less than 1μ in diameter (Fig. 7).

Experiment 5.—Culture 2884-C, Jan. 6. Jan. 9, first passage, Culture 2907-C. Jan. 11, second passage, Culture 2917-C. Jan. 12, camera lucida drawings. Length of cell, 95μ; length of nucleus, 19μ; 30 to 40 red vesicles, 1μ in diameter; a few very small wandering granules, less than 0.5μ in diameter; about 10 small fat granules (Fig. 8).

Subcutaneous Connective Tissue from a Tumor Chicken, Cultivated in Equal Volumes of Plasma and Embryonic Tissue Juice.

Experiment 6.—Culture 3206-C, Feb. 8. Feb. 11, culture stained with cresyl blue; camera lucida drawings. Length of cell, 95μ; length of nucleus, 14μ; a few minute fat granules; 40 to 60 faintly stained vesicles, 0.5 to 1μ in diameter, generally containing 1 small granule, darkly stained; 35 to 40 mitochondria, 3 to 10μ long; a few wandering granules less than 0.5μ in diameter (Fig. 9).

Experiment 7.—Culture 2842-C, Dec. 23, 1925. Dec. 31, first passage, Culture 2856-C. Jan. 2, 1926, second passage, Culture 2859-C. Jan. 4, third passage, Culture 2867-C. Jan. 5, camera lucida drawings. Length of cell, 140μ; length of nucleus, 19μ; 30 to 65 vesicles, 2μ in diameter; about 8 or 10 small wandering granules, 0.5μ in diameter; about 15 small fat globules (Fig. 10). Jan. 7, fourth passage, Culture 2890-C. Jan. 9, camera lucida drawings. Length of cell, 80μ; length of nucleus, 19μ; 40 to 50 red vesicles; 7 to 10 small fat globules; a few small red granules (Fig. 11).

Subcutaneous Connective Tissue from an Adult Chicken, Cultivated in Normal Plasma and Embryonic Juice.

Experiment 8.—Culture 2932-C, Jan. 12. Jan. 22, the tissues were placed in plasma containing a trace of tissue juice until Jan. 29. Jan. 30, Culture 3104-C,

camera lucida drawings. Length of cell, 85μ; length of nucleus, 15μ; 1 to 5 small vesicles, less than 0.5μ in diameter; 10 to 15 elongated vesicles, stained with neutral red, 1.5 to 2μ long; no fat globules; about 10 filamentous mitochondria.

Subcutaneous Connective Tissue from an Adult Chicken, Cultivated in Plasma Containing a Trace of Embryonic Juice.

Experiment 9.—Culture 2892-C, Jan. 7. Jan. 11, first passage in same medium, Culture 2920-C. Jan. 13, camera lucida drawings. Length of cell, 85μ; length of nucleus, 18μ; 7 to 8 neutral red vesicles, 0.25 to 0.5μ in diameter; 4 to 6 neutral red wandering granules, about 0.25μ in diameter; 0 to 8 small fat globules, 0.5μ in diameter (Fig. 12).

In these experiments, a comparative study was made of the number and size of the neutral red vesicles in fibroblasts maintained for 14 years *in vitro* in a condition of optimum activity, and in adult fibroblasts cultivated for periods varying from 24 hours to 13 days, in embryonic tissue juice or in Tyrode solution. The fibroblasts which had proliferated with great activity for 14 years in equal parts of plasma and embryo juice always contained many neutral red vesicles (Fig. 1). Adult connective tissue, on the contrary, cultivated in a similar medium for 24 hours grew very slowly, and the cells contained none or very few vesicles, as shown in Fig. 2. The neutral red vesicles became more numerous and cell proliferation more rapid after 48 hours cultivation in embryonic tissue juice (Figs. 3 to 6). The phenomenon was more evident after 72 hours cultivation (Fig. 7), and after a week (Figs. 8 and 9). When the cells derived from adult connective tissue had been cultivated for a longer time in embryonic tissue juice (Figs. 10 and 11), the number of vesicles was about as great as in the 14 year old strain. However, if embryonic tissue juice was left out of the medium and replaced by Tyrode solution, the activity of the adult connective tissue cells did not increase, and the number of vesicles remained small (Fig. 12).

3. Changes in the Structure of Fibroblasts and the Rate of Growth, Brought About by Variations in the Composition of the Medium.—An attempt was made to ascertain in a more precise manner how the protoplasmic organs are modified by metabolic changes. It is well known that the activity of fibroblasts is affected by the transfer from a nutrient to a non-nutrient medium. If colonies cultivated in plasma

and tissue juice are placed in a coagulum composed almost exclusively of plasma, the rate of growth immediately decreases and proliferation ultimately stops. In the present experiments, the colonies were divided into halves, one being placed in plasma with a trace of tissue juice, and the other in equal parts of plasma and embryo juice. After a few days cultivation, the relations between the rate of growth and the morphological changes could be easily studied.

The fibroblasts were obtained from the subcutaneous connective tissue of adult chickens and prepared from the experiments by being cultivated for a few weeks in plasma and embryonic tissue juice. After their rate of growth and appearance had become analogous to those of the 14 year old strain, the tissues were divided. One half was cultivated in a medium composed of 1 volume of plasma and 1 volume of Tyrode solution containing a trace of embryonic tissue juice, and the other half in equal parts of plasma and embryonic tissue juice. The relative increase of both colonies was measured by a technique previously described.[19] Cinematograph pictures have shown that practically every fibroblast at the edge of a colony moves in straight lines from the center of the colony toward the free medium and that the mechanical activity of the front process is far greater than that of the rear process, which appears to move along, dragged by the body. As the anterior pole of the cells is in a more active condition than the posterior one, it may be assumed that the organs which congregate at the root of the forward processes possess some function related to heightened metabolism. When a marked difference was observed in the rates of growth of the experiment and control tissues, the cells were stained with Janus green and neutral red, and camera lucida drawings made. Ten experiments were performed.

Experiment 1.—30 day old strain of adult connective tissue, Culture 3302-C, Feb. 17. A colony was divided into two parts, one being cultivated in plasma, and the other in 1 volume plasma and 1 volume embryonic juice. Feb. 19, first passage in fresh media; Feb. 20, second passage in fresh media; measurement of the rate of growth and camera lucida drawings.

1. Fibroblasts cultivated in plasma. Rate of growth, 1.1; length of average cell, 120μ; length of nucleus, 18 to 19μ; 20 neutral red vesicles, 0.75μ in diameter, of which more than half were localized in anterior part of the body; 50 mitochondria, 2 to 5μ in length; many fat globules, 0.25 to 1.25μ in diameter in anterior and posterior portions of cell body; about 25 deeply stained neutral red wandering granules, 0.25μ in diameter.

2. Fibroblasts cultivated in plasma and embryonic juice. Rate of growth, 2.3; length of average cell, 130μ; length of nucleus, 21μ; 70 neutral red vesicles, 0.5 to 1μ in diameter in anterior and lateral parts of the body and at root of a large lateral process. A few vesicles contained deeply stained neutral red granules; 60 mitochondria, thin and regular, 3 to 7μ long; 21 fat globules, 0.5μ in diameter; 22 wandering neutral red granules.

Experiment 2.—28 day old strain of adult connective tissue, Culture 3314-C, Feb. 17. Division of a colony into two parts, one being cultivated in plasma, and the other in equal parts of plasma and embryonic juice. Feb. 19, first passage in fresh media; Feb. 21, second passage; Feb. 23, third passage; Feb. 24, camera lucida drawings (Figs. 13 and 14).

1. Fibroblasts in plasma. Rate of growth, 4.6; length of average cell, 110μ; length of nucleus, 15μ; 100 neutral red vesicles, 0.25 to 1μ in diameter, distributed more abundantly in anterior part of the body; no granules in the vesicles; 6 wandering neutral red granules, less than 0.25μ in diameter; 80 mitochondria, 1 to 3μ in length; 20 fat globules.

2. Fibroblasts in plasma and embryonic juice. Rate of growth, 11.0; length of average cell, 100μ; length of nucleus, 16 to 17μ; two-thirds of the mass of neutral red vesicles, about 140 in number, in anterior part of the body, 0.5 to 1.25μ in diameter. 25 fat globules scattered among vesicles; 17 wandering neutral red granules, about 0.25μ in diameter; 70 mitochondria, slender and regular, 6 to 7μ in length.

Experiment 3.—47 day old strain of adult connective tissue, Culture 3331-C, Feb. 17. Division of a colony into two parts, one being cultivated in plasma, and the other in plasma and embryonic tissue juice. Feb. 19, first passage in the same media; Feb. 21, second passage in the same media; Feb. 23, third passage in same media; Feb. 24, camera lucida drawings (Figs. 15 and 16).

1. Fibroblasts cultivated in plasma. Rate of growth, 0.97; length of average cell, 100μ; length of nucleus, 18μ; 50 neutral red vesicles, 0.5 to 0.75μ in diameter, 5 containing small neutral red granules; many small vesicles, less than 0.5μ in diameter; more vesicles in anterior part of the body than in posterior; 80 mitochondria, 1 to 3μ in length; 3 or 4 wandering neutral red granules; no fat globules.

2. Fibroblasts in embryonic juice and plasma. Rate of growth, 2.82; length of average cell, 130μ; length of nucleus, 20μ; 195 vesicles, 0.25 to 1μ in diameter, more being located in anterior part of cells than in posterior; many very small neutral red vesicles scattered throughout cytoplasm; about 35 wandering neutral red granules in the cell processes; 90 mitochondria, 3 to 9μ in length; 35 small fat globules.

Experiment 4.—41 day old strain of adult fibroblasts, Culture 3253-C, Feb. 10. Fragment divided into two parts, one being cultivated in plasma, and the other in plasma and embryonic tissue juice. Feb. 12, first passage in same media; Feb. 13, second passage in same media; Feb. 15, third passage in same media; Feb. 16, camera lucida drawings (Figs. 17 and 18).

1. Fibroblasts in plasma. Rate of growth, 1.2; length of average cell, 90μ; length of nucleus, 16μ; 22 neutral red vesicles, 0.25 to 0.75μ in diameter, located in anterior part of the body; 26 wandering neutral red granules; 75 fat globules located in posterior part; 90 mitochondria, 5 to 10μ in length.

2. Fibroblasts in plasma and embryonic juice. Rate of growth, 4; length of average cell, 80μ; length of nucleus, 16μ; 135 neutral red vesicles, 0.5 to 1μ in diameter, located in anterior and lateral parts of body at root of large side process; 14 small fat globules in posterior part; 60 mitochondria, 5 to 8μ in length.

Experiment 5.—19 day old strain of adult fibroblasts, Culture 3531-C, Mar. 15. Fragment divided into two parts, one being cultivated in plasma, and the other in equal volumes of plasma and embryonic juice. Mar. 18, first passage into same media; Mar. 19, camera lucida drawings.

1. Fibroblasts in plasma. Rate of growth, 1.5; length of average cell, 105μ; length of nucleus, 19μ; 16 neutral red vesicles, 0.25 to 0.50μ in diameter, located in anterior part of the cell body; 36 filamentous mitochondria, 5 to 15μ in length; 47 small fat globules.

2. Fibroblasts in plasma and embryonic juice. Rate of growth, 10.9; length of average cell, 110μ; length of nucleus, 19.5μ; 140 neutral red vesicles, 0.25 to 1.5μ in diameter, located in anterior part of the cell body lateral to the nucleus; disposition of most of the vesicles around centriole; 40 mitochondria, 3 to 6μ in length; 24 fat globules; a group of larger ones posterior to the nucleus.

Experiment 6.—14 year old strain of fibroblasts, Culture 3068-A, Feb. 15. A fragment was divided into two parts, which were cultivated in two flasks in the ordinary medium. Feb. 16, both cultures were washed and patched. Feb. 17, both cultures were washed for 10 minutes with Tyrode solution, then 0.5 serum and 0.5 Tyrode solution were introduced into Flask A, and 1 cc. of pure embryo extract into Flask B. Feb. 19, both cultures were washed and the same media introduced. Feb. 20, growth had stopped in Flask B, while it was still rapid in Flask A. Measurement of the area covered by the fibroblasts, transfer of the tissues to cover-glasses, and camera lucida drawings.

1. Fibroblasts cultivated in Tyrode solution. Growth stopped; length of average cell, 90μ; length of nucleus, 13 to 15μ; 8 neutral red vesicles, 0.25 to 0.5μ in diameter; 10 wandering neutral red granules; 50 mitochondria, 1 to 2μ in length; 54 fat globules around nucleus and posterior to it.

2. Fibroblasts cultivated in embryonic juice. Rate of growth, 3; length of average cell, 110μ; length of nucleus, 18μ; 42 neutral red vesicles, 0.25 to 1.25μ in diameter; 14 wandering neutral red granules; 50 mitochondria, 2 to 7μ in length; 46 fat globules around nucleus and posterior to it.

Experiment 7.—36 day old strain of adult connective tissue, Culture 3348-C. A fragment was divided into two parts, one being cultivated in 3 volumes Tyrode solution and 1 volume plasma, and the other in 3 volumes embryonic juice and 1 volume plasma. Feb. 26, camera lucida drawings (Figs. 19 and 20).

1. Fibroblasts in Tyrode solution. Rate of growth, 0.77; length of average cell. 120μ; length of nucleus, 17μ; 14 neutral red vesicles, 1μ in diameter; 15 wandering neutral red granules; 100 mitochondria, 1 to 2μ in length; no fat globules.

2. Fibroblasts in embryonic juice. Rate of growth, 3.28; length of average cell, 140μ; length of nucleus, 22μ; 195 neutral red vesicles, 0.5 to 1μ in diameter; 10 vesicles with neutral red granules; 63 mitochondria, 2 to 9μ in length; no fat globules.

Experiment 8.—14 year old strain of fibroblasts, Culture 3303-C, Feb. 15. Two flasks were prepared, each containing four fragments of the old strain, cultivated in ordinary medium. Feb. 16, both cultures washed; 1 cc. of Tyrode solution was introduced into Flask A, and 1 cc. of embryonic juice into Flask B. Feb. 17, both cultures washed in Tyrode solution and given fresh media; measurement of relative increase; Feb. 18, same treatment; Feb. 19, measurement of increase, transfer of cultures to glass slides, and camera lucida drawings (Figs. 21 and 22).

1. Fibroblasts in Tyrode solution. Rate of growth, 1; length of average cell, 100μ; length of nucleus, 11μ; 49 neutral red vesicles, 0.25 to 0.5μ in diameter; 5 wandering neutral red granules; 60 mitochondria, 0.25 to 2μ in length; 50 fat globules.

2. Fibroblasts in embryonic juice. Rate of growth, 7; length of average cell, 120μ; length of nucleus, 22μ; 190 neutral red vesicles, 0.25 to 1μ in diameter; 15 wandering neutral red granules; 50 mitochondria, 1 to 5μ in length; 18 fat globules.

Experiment 9.—Culture 3502-C, Mar. 1. A fragment of chick embryo heart was divided into two parts, one being cultivated in 1 volume plasma and 3 volumes Tyrode solution containing a trace of embryonic juice, and the other in 1 volume plasma and 3 volumes embryonic juice. In the course of 16 days, both fragments were transferred six times into fresh media. Mar. 17, measurement of rate of growth, and camera lucida drawings.

1. Fibroblasts cultivated in Tyrode solution. Rate of growth, 0.03; length of cell, 90μ; length of nucleus, 11μ; 5 neutral red vesicles, 0.25μ in diameter; 2 wandering red granules; 20 mitochondria, 1 to 2μ in length; 95 small fat globules around the nucleus.

2. Fibroblasts cultivated in embryonic juice. Rate of growth, 3.0; length of cell, 120μ; length of nucleus, 18μ; 195 neutral red vesicles, 1 to 2μ in diameter; 13 wandering red granules; 50 mitochondria, 3 to 6μ in length; 45 very small fat globules.

Experiment 10.—Culture 3623-C, Feb. 21. 8 day old strain of fibroblasts. Mar. 1, fragment was divided into two parts, one being cultivated in 1 volume plasma and 3 volumes Tyrode solution containing a trace of embryonic juice, and the other in 1 volume plasma and 3 volumes embryonic juice. In the course of 29 days, both fragments were transferred fifteen times into fresh media. Mar. 30, measurement of rate of growth, and camera lucida drawings (Figs. 23 and 24).

1. Fibroblasts cultivated in Tyrode solution. Rate of growth, 0.01; length of cell, 75μ; length of nucleus, 11μ; 11 neutral red vesicles, 0.25μ in diameter; 5 wandering red granules; 39 mitochondria, 2 to 5μ in length; no fat globules.

2. Fibroblasts cultivated in embryo juice. Rate of growth, 2.5; length of cell, 110μ; length of nucleus, 15μ; 100 neutral red vesicles, 0.25 to 1.5μ in diameter; 13 wandering red granules; 55 mitochondria, 2 to 12μ in length; 45 small fat globules.

In these ten experiments, fibroblasts in a known condition of activity and showing long filamentous mitochondria and a number of small neutral red vesicles (Figs. 14, 16, 18, 20, 22, and 24) were cultivated in media deprived of embryonic juice and composed chiefly of serum or Tyrode solution. Their rate of proliferation immediately decreased and at the same time their structure underwent marked changes. The cytoplasmic organs of the cells living in plasma alone assumed the appearance of those of resting fibroblasts (Figs. 13, 15, and 17). The granules disappeared from the vesicles. The neutral red vesicles were reduced to one-half or one-third of their normal number. At the same time, the size of the nucleus decreased, and the mitochondria became shorter. No modification took place in the controls, which were maintained in embryonic juice. The simultaneous variations of the rate of growth and of the protoplasmic organs are summarized in Table I. When the colonies were cultivated in Tyrode solution, the changes became more apparent (Figs. 19, 21, and 23). The decrease in the size of the nucleus was greater. For instance, in Experiment 8, where cell proliferation had completely stopped, the nucleus was only 11μ in length while it was 22μ in the control (Figs. 21 and 22). The mitochondria were not greatly modified in number but, instead of being filaments 5 to 6μ in length, they had become very short rods or dots. The fat globules were much more abundant. The neutral red vesicles were reduced to one-tenth of the number present in the control. They were smaller and contained no granules (Table I). The results of both series of experiments may be summarized as follows:

The significance of the neutral red vesicles observed in normal cells has been investigated by studying fibroblasts artificially placed in definite states of activity. The lowest metabolic condition was obtained by cultivating fragments of subcutaneous connective tissue, removed from an adult chicken, in a medium composed almost exclusively of plasma. The first cells which slowly migrated from the fragment were almost in a resting condition. After a few days cultivation in plasma and embryonic juice, their activity increased, as shown by measurement of their rate of growth which finally became equal to

TABLE I.

Relation between the Rate of Growth and the Number and Size of Cell Structures.

Experiment No.	Culture No.	Fig. No.		Rate of growth.		Length of cell.		Length of nucleus.		No. of neutral red vesicles.		Diameter of neutral red vesicles.		No. of mitochondria.		Length of mitochondria.		No. of fat globules.		No. of wandering neutral red granules.	
		Plasma.	Tissue juice.	Plasma.	Tissue juice.	Plasma.	Tissue juice.	Plasma.	Tissue juice.	Plasma.	Tissue juice.	Plasma.	Tissue juice.	Plasma.	Tissue juice.	Plasma.	Tissue juice.	Plasma.	Tissue juice.	Plasma.	Tissue juice.
						μ	μ	μ	μ			μ	μ			μ	μ				
1	3302-C			1.1	2.3	120	130	18–19	21	20	70	0.75	0.5–1	50	62	2–5	3–7	60	21	25	22
2	3314-C	13	14	4.6	11.0	110	110	15	15–17	100	140	1	1	80	70	1–3	6–7	20	25	6	17
3	3331-C	15	16	0.97	2.82	100	130	18	20	50	195	0.5–0.75	0.25–1	80	90	1–3	3–9	0	35	3–4	35
4	3253-C	17	18	1.2	4.0	90	80	16	16	22	135	0.25–0.75	0.5–1	90	60	5–10	5–8	75	14	26	13
5	3531-C			1.5	10.9	105	110	19	19.5	16	140	0.25–0.5	0.25–1.5	36	40	5–15	3–6	47	24	5	9
		Tyrode.		Tyrode.		Tyrode.		Tyrode.		Tyrode.		Tyrode.		Tyrode.		Tyrode.		Tyrode.		Tyrode.	
6	3068-A			0.0	3.0	90	110	13–15	18	8	42	0.25–0.5	0.25–1	50	50	1–2	2–7	54	46	10	14
7	3348-C	19	20	0.77	3.28	120	140	17	22	14	195	1	0.5–1	100	63	1–2	2–9	0	0	15	35
8	3303-C	21	22	1.0	7.0	100	120	11	22	18	190	0.25–0.5	0.25–1	60	50	0.2–2	1–5	50	18	5	15
9	3502-C			0.03	3.0	90	120	11	18	5	195	0.25	1–2	20	50	1–2	3–6	95	45	1	13
10	3623-C	23	24	0.01	2.5	75	110	11	15	11	100	0.25	0.25–1.5	39	55	2–5	2–12	0	45	5	13

that of a pure strain of fibroblasts cultivated for 14 years in plasma and embryonic juice. The effects of decreased metabolism on protoplasmic structures were observed in a precise manner by depriving fibroblasts, which had reached their optimum rate of growth, of embryonic tissue juice and cultivating them in Tyrode solution and also in serum or plasma. The relations between the metabolic conditions as expressed by the rate of proliferation and the cell structure immediately became clear.

At the beginning of their life *in vitro*, the adult fibroblasts, and embryonic fibroblasts cultivated in plasma or in Tyrode solution until their activity had decreased or stopped, presented some common characteristics. Their cytoplasm showed very few small neutral red vesicles. These vesicles did not contain any granules. There were no fat globules in the body of the cell, and the mitochondria consisted of short filaments. Simultaneously with the increase of the rate of growth obtained by cultivation in embryonic juice, the neutral red vesicles became more numerous and the filamentous mitochondria longer. Small red bodies appeared within the vesicles, and a number of wandering granules moved through the process and the body. When the cells had reached their highest activity, the number of the vesicles became maximum, as well as the length of the mitochondria. The phenomenon was reversible. If colonies of the 14 year old strain of fibroblasts were placed in serum or in Tyrode solution, the rate of growth decreased and at the same time the vesicles lost their granules and were reduced in number, while the mitochondria became much shorter. The neutral red vesicles congregated in the anterior part of the body, that is, at the active pole. If a lateral process developed and became more active, the vesicles shifted to the root of this process. In either case, they were probably grouped around the centriole, as observed by Lewis.[20] But in normal fibroblasts, the centriole generally remains invisible. It was obvious that the number and size of the vesicles were related to metabolism and not to the duration of cultivation. Fibroblasts obtained from adult connective tissue and from the 14 year old strain, and cultivated in embryo juice, assumed the same appearance as soon as their respective rates of growth had become

[20] Lewis, W. H., *Anat. Rec.*, 1919, xvi, 155.

similar. This fact shows that the morphology of a fibroblast does not depend on its origin, adult or embryonic, but on the presence and the concentration of certain substances in the pericellular fluid. The length of the cell, the size of the nucleus, the number, size, and content of the neutral red vesicles, the number of the wandering red granules, and the size of the mitochondria vary with the rate of growth of the colony and the composition of the medium (Table I). Then the structure of the cells can be modified at will, and the appearance that they will assume when living in media of definite composition may be accurately predicted. It has become possible to ascertain the physiological state of a fibroblast from its anatomical characteristics. The appearance of the cytoplasmic organs indicates whether a fibroblast is at rest, in a condition of moderate or optimum activity, or in a starving condition. The active pole of the cell and the direction of its migration can also be detected by the location of the neutral red vesicles.

DISCUSSION.

The determination of the specific physiological properties of the fibroblast is of evident importance, as the individuality of a cell depends on its functions far more than on its staining reactions. The morphological analysis of a protoplasmic structure merely leads to its identification. When a bacterium is only characterized by its shape and mode of reaction toward dyes, our knowledge of it is of an unsatisfactory nature, and must be completed by the study of its properties in pure cultures, such as its action on the medium, the food it requires for multiplication, the poisons it secretes, its susceptibility to various chemical substances, etc. The same is true of tissue cells. The most minute anatomical descriptions are without great interest as long as morphology is not correlated with function, and as the essential properties of the cell remain unknown. Through the foregoing experiments, the fibroblast has become characterized not only by the structure of its nucleus and of its mitochondria and neutral red vacuoles, but also by the appearance of its colonies, its action on the medium, its relation to other cells, its rate of proliferation under given conditions, its mode of locomotion, the movements of its cytoplasmic organs, the food it requires for multiplication, etc. This new knowledge has made it possible to understand and to foresee the behavior of

the cell within the organism. It became obvious that fibroblasts must be in a resting condition within the adult animal on account of their inherent property of requiring for multiplication substances which are not present in lymph or blood serum. It is also evident that they are capable of resuming their embryonic activity, even in extreme old age, during the process of wound healing, or of organ sclerosis, because they have the power of feeding on substances set free by leucocytes or by epithelial cells, as happens when adult connective tissue is cultivated in embryonic juice. These and other working hypotheses may be constructed on the knowledge of the biological properties of the fibroblast, in order to explain, partly at least, the mechanism of the complex phenomena in which connective tissue cells participate.

The study of pure strains of fibroblasts brought into definite states of activity by cultivation in various media, under such conditions that they do not degenerate, has led to a correlation of certain protoplasmic structures and nutritional conditions. Neutral red vesicles and their granules are evidently connected with cell metabolism. The vesicles are more numerous while the colonies develop rapidly; they lose their granules and decrease in number if proliferation becomes slower or stops after the colonies have been deprived of food. They appear to be organs of great importance, the development of which is intimately bound up with the physiological state of the cell. Evidently they elaborate substances which are either used by the cells in their rapid proliferation, or accumulated as reserves, and possibly as material for exogenous secretion. Whether stored up foodstuff, or secretory material, these substances are the result of protoplasmic activity, and are manufactured within vesicles which must be considered as the normal organs of the active fibroblast. These vesicles differ markedly from the vacuoles that develop around bacteria and foreign bodies,[1,21] and from the degeneration vacuoles described by Lewis.[5] Neutral red is not a specific stain. It colors normal and degenerating structures in the same manner. The presence in the connective tissue cells of different types of vacuoles was more clearly understood by the earlier anatomists than by the modern ones. The distinction between the secretory vacuoles and those that develop around a foreign body was

[21] Prigosen, R. E., *Bull. Johns Hopkins Hosp.*, 1921, xxxii, 206.

made long ago by Renaut in his masterly investigations of the connective tissue cells.[1] The degeneration vacuoles remained unknown to him because his observations were made exclusively on tissues freshly extirpated from the animal. The degeneration vacuoles are a modern product of defective techniques and develop in cells which are erroneously believed to be in a condition of cultivation. The neutral red vesicles observed by Renaut[1] in the fibroblast and the macrophage were identical with those described as the normal segregation apparatus by Evans and Scott[2] in their excellent study of the connective tissue cells, and with those observed by us at every stage of their development *in vitro*.

In the early observations by Renaut, the vesicles assumed various characteristics according to the age of the animals from which the tissues were obtained. Very few were observed in the normal connective tissue of adult animals. But in fetuses or very young animals, every fibroblast presented some red vacuoles containing a segregation granule. The number of the stained cells was found to decrease with advancing age and in the old animal they almost completely disappeared. Renaut concluded that the presence of the neutral red vesicles is a characteristic of the young fixed connective tissue cell.[1] He corroborated these findings by a study of the tendons of the tail of the rat, mouse, rabbit, and cat. In young animals, every fibroblast was seen to contain neutral red vesicles, while in older animals, none of the cells stained red. The presence of the vesicles was evidently related to a function of the fibroblast which persisted as long as the tendon fibers were not definitely organized. Young cells and cells in a condition of secretory activity appeared to be endowed with the property of elaborating segregation granules within the fluid of vesicles. Renaut gave to those cells the name of rhagiocrin, from ῥαγιον, grape, because granules growing in a vesicle resemble the seeds of a grape. The segregation apparatus of the fibroblast and the macrophage was considered by him as a common characteristic of both cells, the macrophage having an apparatus very much more developed than the fibroblast.

The results of our experiments are in agreement with this early conception of the functions of the neutral red vacuoles. It is certain that they represent an organ which characterizes cells in a condition of metabolic activity, and that their development may be increased or decreased at will by the substances which modify the rate of growth. The normal segregation apparatus of the fibroblast differs from both degeneration vacuoles and vacuoles formed around foreign bodies.

The length and number of the mitochondria have been found to

undergo some variations which are definitely related to those of cell metabolism. In the more active colonies, they are long filamentous organs from 5 to 10μ in length, which present the morphological characteristics minutely described by Lewis and Lewis.[4] The irregular and abnormal forms, which they also mentioned, were rarely observed. It is probable that they are more often present in cells that begin to degenerate. The normal mitochondria were seen to become shorter and slightly thicker when the rate of growth of the colonies decreased. They broke down into dots in cells starved in Tyrode solution. If the activity of the cells was increased by cultivation in embryonic juice, the mitochondria again became longer. Their number did not vary greatly. Their mode of distribution through the body and the active and less active processes remained approximately constant. They did not congregate in the region of the cell which displayed the greatest mechanical activity, as neutral red vesicles do. Mitochondria are certainly endowed with a function more fundamental to the life of the cell than the neutral red vesicles, because they always remain present in resting cells, when the neutral red vesicles have almost completely disappeared. So far, it has generally been believed, as expressed by Cowdry,[22] that mitochondria possess a fundamental rôle in cell activity. But the nature of this rôle is not as yet clearly understood. Lewis[4] thought that the quantity of mitochondria differs so widely that it is impossible to connect it with any definite function. He believed that these great variations depended on the metabolism of the individual cells. The truth of this hypothesis was demonstrated by our experiments. All the fibroblasts which compose the outer edge of a growing colony are in identical metabolic condition. The appearance of the cells becomes uniform. There is but little variation in the size and quantity of the mitochondria in the fibroblasts of a given culture. However, when the activity of the whole colony is affected by a change in the medium, the mitochondria of every visible cell undergo parallel modifications.

From the foregoing experiments, it may be concluded that:

1. The fibroblast is characterized not only by its morphology and

[22] Cowdry, E. V., *Carnegie Institution of Washington, Pub. No. 271, Contributions to Embryology*, 1919, viii, 39.

staining reaction, but also by a number of physiological properties, which become apparent in the pure cultures of the cell.

2. There is a definite relation between the size, the number, and the form of certain cytoplasmic structures and the metabolic conditions of the fibroblast.

EXPLANATION OF PLATES.

PLATE 6.

FIG. 1. Fibroblasts cultivated for 14 years in equal parts of plasma and embryonic juice. Camera lucida drawing made 24 hours after passage into fresh medium.

FIG. 2. Fibroblasts from subcutaneous connective tissue of young adult chicken, cultivated in equal volumes of plasma and embryonic juice. Camera lucida drawing made after 24 hours incubation.

FIGS. 3 and 4. Fibroblasts from subcutaneous connective tissue of an adult chicken, cultivated for 48 hours in equal volumes of plasma and embryonic juice. Camera lucida drawing made after 48 hours incubation.

FIGS. 5 and 6. Fibroblasts from subcutaneous connective tissue of an adult chicken, cultivated for 48 hours in equal volumes of plasma and embryonic juice. Camera lucida drawing made after 48 hours incubation.

FIG. 7. Fibroblasts from subcutaneous connective tissue of an adult chicken, cultivated for 72 hours in equal volumes of plasma and embryonic juice. Camera lucida drawing made 24 hours after first passage into fresh medium.

FIG. 8. Fibroblasts from subcutaneous connective tissue of an adult chicken, cultivated for 5 days in equal volumes of plasma and embryonic juice. Camera lucida drawing made 24 hours after second passage into fresh medium.

FIG. 9. Fibroblasts from subcutaneous connective tissue of a tumor chicken, cultivated for 72 hours in equal volumes of plasma and embryonic juice. Camera lucida drawing made after 24 hours incubation.

FIG. 10. Fibroblasts from subcutaneous connective tissue of a tumor chicken, cultivated for 13 days in equal volumes of plasma and embryonic juice. Camera lucida drawing made 24 hours after third passage into fresh medium.

FIG. 11. Fibroblasts from same strain of connective tissue as shown in Fig. 10, cultivated for 17 days in equal volumes of plasma and embryonic juice. Camera lucida drawing made after 48 hours incubation.

FIG. 12. Fibroblasts from subcutaneous connective tissue of an adult chicken, cultivated for 6 days in plasma containing a trace of embryonic juice. Camera lucida drawing made after 48 hours incubation.

PLATE 7.

FIG. 13. Culture 3314-C. Fibroblasts in plasma. Rate of growth, 4.6.

FIG. 14. Culture 3314-C. Fibroblasts in plasma and embryonic juice. Rate of growth, 11.0.

FIG. 15. Culture 3331-C. Fibroblasts in plasma. Rate of growth, 0.97.

FIG. 16. Culture 3331-C. Fibroblasts in plasma and embryonic juice. Rate of growth, 2.82.

FIG. 17. Culture 3253-C. Fibroblasts in plasma. Rate of growth, 1.2.

FIG. 18. Culture 3253-C. Fibroblasts in plasma and embryonic juice. Rate of growth, 4.0.

FIG. 19. Culture 3348-C. Fibroblasts in Tyrode solution. Rate of growth, 0.77.

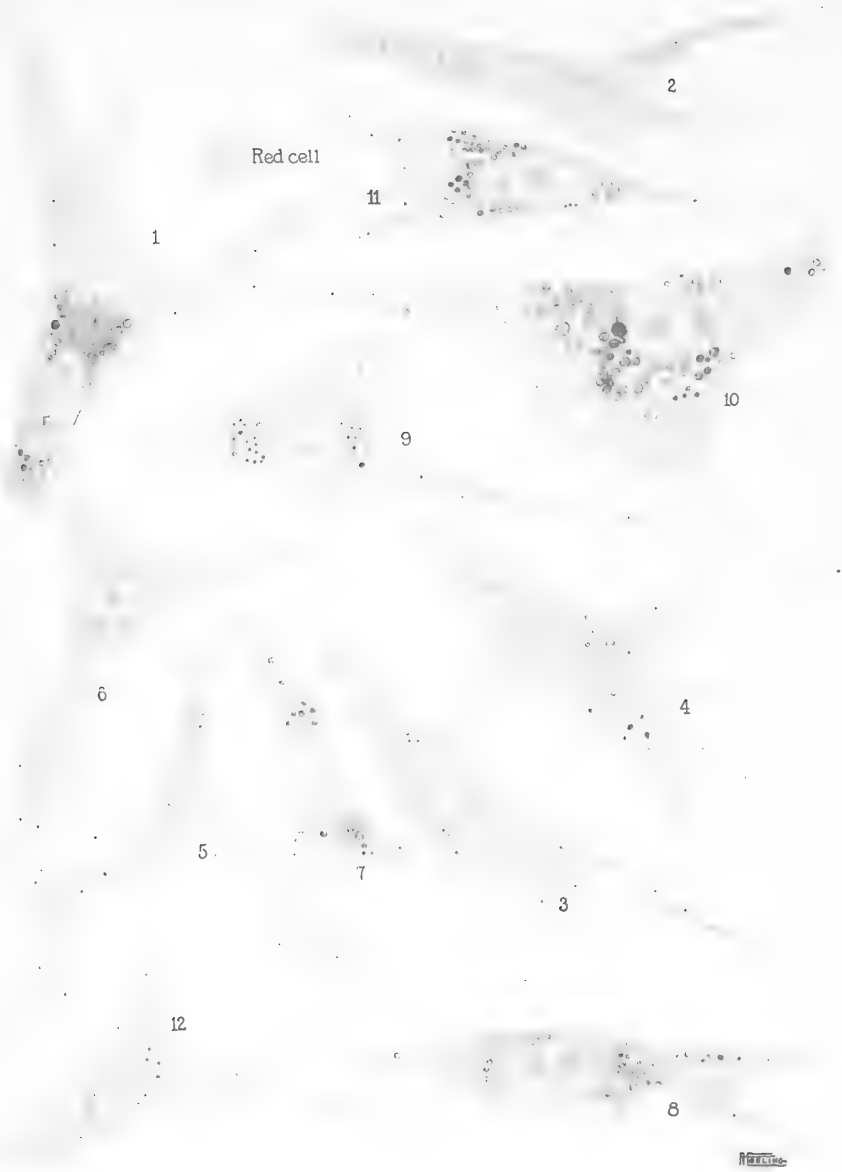
FIG. 20. Culture 3348-C. Fibroblasts in embryonic juice. Rate of growth, 3.28.

FIG. 21. Culture 3303-C. Fibroblasts in Tyrode solution, from same experiment. One stained with neutral red and the other with Janus green. Rate of growth, 1.0.

FIG. 22. Culture 3303-C. Fibroblasts in embryonic juice, one stained with neutral red and the other with Janus green. Rate of growth, 7.0.

FIG. 23. Culture 3623-C. Fibroblasts in Tyrode solution. Rate of growth, 0.01.

FIG. 24. Culture 3623-C. Fibroblasts in embryonic juice. Rate of growth, 2.5.



(Carrel and Ebeling: Properties of fibroblast and macrophage. I.)

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